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THE BIOSYNTHESIS OF GLYCINE AND SERINE BY ISOLATED CHLOROPLASTS

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Abstract—Chloroplasts were isolated non-aqueously from the leaves of 15-day-old pea plants. After sonication aqueous extracts were assayed for ability to synthesize glycine and serine. Such extracts were found to contain glyoxylate aminotransferase activity which was stimulated by addition of pyridoxal-5′-phosphate. Boiled enzyme controls indicated that the reaction was mainly enzymic. Chloroplast extracts also catalyzed the synthesis of serine from glycine. The reaction displayed requirements for tetrahydrofolate (H_4PteGlu) and pyridoxal-5′-phosphate and was further stimulated by additions of formaldehyde. Sonicated chloroplasts also catalyzed the incorporation of [^{14}C]-formaldehyde into serine in a reaction having requirements for glycine, H_4PteGlu and pyridoxal-5′-phosphate. [3-^{14}C]-serine was readily converted into [^{14}C]-formaldehyde by chloroplast extracts with similar reaction requirements. On the basis of these assays it is concluded that chloroplasts contain a serine hydroxymethyltransferase (L-serine: tetrahydrofolate-5,10-hydroxymethyltransferase EC 2.1.2.1).

INTRODUCTION

IT IS now well established that glycine and serine are readily formed and metabolized by photosynthetic tissues.[1–13] The synthesis of glycine is thought to include glycollate oxidase (glycollate: oxygen oxidoreductase EC 1.1.3.1) and glyoxylate aminotransferase catalyzed reactions as glycollate and glyoxylate are both readily converted into this amino acid.[2,6,8,10,14] The synthesis of serine in such plants appears to involve serine hydroxymethyltransferase activity as suggested by the observation[2] that serine is uniformly labelled after short periods of photosynthesis in $^{14}CO_2$. In addition there is evidence that serine may also be formed by aminotransferase reactions involving hydroxypyruvate and phosphohydroxypyruvate.[15–19]

Investigations have been conducted to determine the intracellular sites of synthesis of

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various photosynthetic products. These studies have examined the distribution of radioactivity between chloroplastic and non-chloroplastic fractions of leaves after periods of photosynthesis in $^{14}CO_2$.[4,5,20] From such experiments it is clear that a rapid exchange of materials occurs between the chloroplast and other parts of the cell. Both glycine and serine were very rapidly labelled in experiments with tobacco leaves[5] and both compounds occurred in chloroplastic and non-chloroplastic pools. However as kinetic studies of this incorporation of ^{14}C were not conducted it is difficult to determine whether these amino acids were synthesized initially within the chloroplast or were derived from labelled intermediates transported out of the chloroplast.

It is possible that in photosynthesizing cells synthesis of amino acids may occur in more than one cellular compartment and furthermore that different sites of synthesis may assume different degrees of importance as the leaf ages. For example, Mukerji and Ting[21] have shown that aspartate aminotransferase activity is detectable in both the mitochondria and chloroplasts of *Opuntia*. In recent work Tolbert's group[22] have shown that glycine biosynthesis is readily catalyzed by isolated leaf peroxisomes. In work with young maize seedlings Shah and Rogers[23] have presented evidence for generation of acetyl CoA from glycollate via the intermediary formation of glycine and serine. The pathway was shown to have importance in the biosynthesis of plastid terpenoids and the results of feeding experiments were consistent with localization of the reactions within the chloroplast. It was suggested that generation of acetyl CoA by this route would be of greatest significance in young seedlings synthesizing terpenoids and that the pathway may be of less importance with the onset of leaf senesence.[24]

Recent work[25,26] from this laboratory has demonstrated that tetrahydrofolate (H_4PteGlu) derivatives occur in isolated chloroplasts. This finding suggests that chloroplasts may be the site of various one-carbon transfer reactions including the biosynthesis of serine from glycine. As outlined above, reports in the literature suggest that chloroplasts contain pools of these amino acids but there have been no reports, to our knowledge, of studies on the enzymes catalyzing these syntheses in isolated chloroplasts. The present investigation was therefore carried out with chloroplasts isolated from young pea seedlings in order to examine their ability to synthesize glycine and serine and to determine the nature of the reactions involved.

RESULTS

(1) *Glyoxylate Aminotransferase Activity in Chloroplast Extracts*

In a preliminary study with chloroplast extracts, it was evident that a part of the radioactivity appearing in glycine from [2-^{14}C]-glyoxylate was due to a non-enzymic transamination reaction. Consequently, a boiled enzyme preparation was included as a control in each experiment. When chloroplast extracts were assayed for glyoxylate aminotransferase activity (Table 1) it was clear that significantly more labelled glycine was formed in the presence of unboiled enzyme. Three L-amino acids were studied as possible amino group donors. It is evident that L-glutamate, L-aspartate and L-alanine are all able to carry out this function,

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although L-glutamate appears to be the most effective. The reaction was also affected by omission of pyridoxal-5′-phosphate and to a lesser extent by omission of $MgSO_4$.

TABLE 1. GLYOXYLATE AMINOTRANSFERASE ACTIVITY IN ISOLATED PEA CHLOROPLASTS

Omission from complete reaction system	[^{14}C]-Glycine (dpm/mg protein × 10^{-3})	% Of boiled enzyme control
None (boiled enzyme)	13·8	100
None	36·5	264
Pyridoxal-5′-phosphate	2·2	16
$MgSO_4$	24·4	177
L-Glutamate	2·1	15
L-Glutamate*	25·2	182
L-Glutamate†	19·6	142

* 2·0 μM L-aspartate as amino donor.

† 2·0 μM L-alanine as amino donor. The complete system contained L-glutamate, 2·0 μM; pyridoxal-5′-phosphate, 8·0 μM; $MgSO_4$, 8·0 μM; [2-^{14}C]-glyoxylate, 0·125 μM (16·0 μc/μM) and K-phosphate buffer (pH 7·4), 15·0 μM. This mixture was preincubated at 30° for 5 min followed by initiation of the reaction by addition of 0·5 ml of chloroplast extract (1–2 mg protein). Total vol. 1·5 ml.

(2) *Synthesis of Serine from Glycine by Chloroplast Extracts*

Chloroplast extracts were also found capable of catalyzing the synthesis of serine from glycine as shown in Table 2. This synthesis did not occur in the absence of the plant extract and was markedly stimulated by additions of pyridoxal-5′-phosphate and H_4PteGlu. A further, but smaller, stimulation was observed when formaldehyde was added. In other assays involving [^{14}C]-formaldehyde it was clear that this compound was incorporated into serine in a reaction which was dependent on the presence of glycine, pyridoxal-5′-phosphate and H_4PteGlu (Table 2).

TABLE 2. SYNTHESIS OF SERINE FROM GLYCINE BY CHLOROPLAST EXTRACTS

Reaction system	[^{14}C]-Serine (dpm/mg protein × 10^{-3})
[^{14}C]-Glycine → [^{14}C]-Serine	
Complete*	48·8
Minus enzyme	nil
Minus pyridoxal-5′-phosphate	2·4
Minus H_4PteGlu	1·8
Plus formaldehyde (6·0 μM)	53·9
[^{14}C]-HCHO → [^{14}C]-Serine	
Complete†	41·5
Minus glycine	nil
Minus H_4PteGlu	2·1
Minus pyridoxal-5′-phosphate	2·0

* The complete reaction system contained H_4PteGlu, 10·0 μM; pyridoxal-5′-phosphate, 8·0 μM; [2-^{14}C]-glycine, 0·15 μM (21·8 μc/μM) and K-phosphate buffer (pH 7·4). After preincubation of this mixture at 30° for 5 min, the reaction was initiated by addition of chloroplast extract containing 1–2 mg protein. Total vol. 1·5 ml.

† 0·2 μM of formaldehyde (22·6 μc/μM) were added to the complete system containing 3·0 μM unlabelled glycine.

(3) *Serine Hydroxymethyltransferase Activity in Isolated Chloroplasts*

In studies of serine hydroxymethyltransferase, employing L-[3-^{14}C]serine as a substrate (Table 3) it was clear that the isolated chloroplasts contained appreciable levels of this enzyme. The reaction was found to have absolute requirements for both pyridoxal-5′-phosphate and H_4PteGlu. In further experiments to study the specificity of the enzyme for H_4PteGlu and H_4PteGlu$_3$ (Table 3) both compounds were found to be effective as acceptors of the the β-carbon of serine. However, the radioactivity appearing in the HCHO-dimedone derivative from L-[3-^{14}C]-serine in the presence of H_4PteGlu$_3$ was only 10 per cent of that observed when H_4PteGlu was used.

TABLE 3. CONVERSION OF [3-^{14}C]-SERINE TO [^{14}C]-FORMALDEHYDE BY CHLOROPLAST EXTRACTS

Reaction system	dpm/mg Protein in HCHO-dimedone × 10^{-3}	
	H_4PteGlu	H_4PteGlu$_3$
Complete	24·2	3·4
Minus enzyme	nil	nil
Minus pyridoxal-5′-phosphate	2·2	1·7
Minus H_4PteGlu	3·4	—
Minus H_4PteGlu$_3$	—	2·9

DISCUSSION

On the basis of the data presented in Table 1 it is clear that non-aqueously isolated chloroplasts contain appreciable levels of glyoxylate aminotransferase activity. In agreement with earlier work[27,28] the synthesis of glycine was found to proceed, in part, non-enzymically. The considerable stimulation in glycine formation on addition of an amino acid suggests that the reaction involves amino transfer. This possibility is also indicated by the requirement for pyridoxal-5′-phosphate (Table 1) which has been noted by other workers for glyoxylate aminotransferases from other species.[29,30] The present results also suggest that the chloroplastic enzyme, in common with other glyoxylate aminotransferases studied to date,[22,30–33] will utilize more than one amino donor. Although glutamate was the most effective amino donor in the present studies (Table 1), the activities shown on addition of alanine and aspartate could reflect either a broad substrate specificity or alternatively the existence in chloroplasts of more than one glyoxylate aminotransferase.

The synthesis of serine by isolated chloroplasts which was glycine, H_4PteGlu and pyridoxal-5′-phosphate dependent (Table 2) supports the suggestion that chloroplasts contain serine hydroxymethyltransferase. Direct evidence for the occurrence of this enzyme in non-aqueously isolated chloroplasts is given by the data in Table 3. The considerable labelling of serine in the absence of an added one-carbon donor (Table 2) is surprising, but, as these particular chloroplasts were not dialyzed, could reflect an exchange reaction with serine in the

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chloroplastic pool. The stimulation of serine labelling when formaldehyde was added to the reaction system and the incorporation of [^{14}C]-formaldehyde in a reaction dependent on pyridoxal-5′-phosphate, glycine and H_4PteGlu are all consistent with the presence of serine hydroxymethyltransferase in chloroplasts. The greater utilization of H_4PteGlu rather than H_4PteGlu$_3$ in the reaction (Table 3) may be of physiological significance as triglutamate derivatives of tetrahydrofolic acid are apparently not present in pea chloroplasts isolated aqueously or non-aqueously.[34]

Considering the role of these enzymes in metabolism of the chloroplast it is clear that operation of the proposed route for acetyl CoA synthesis[14,23] requires that chloroplasts have ability to catalyze the conversion of glyoxylate → glycine → serine. Such ability is clear from the present work and data from feeding and inhibitor studies conducted by Shah *et al.*[12,35] are consistent with operation of this reaction sequence. The glycollate pathway[36] leading to the synthesis of carbohydrate has several reactions in common with the proposed acetyl CoA pathway; however, recent evidence[37] suggests that the former pathway occurs largely outside the chloroplast. The possible contamination of the chloroplasts used in the present studies by cytoplasmic enzymes would be expected to be minimal[38] as the particles were washed several times with organic solvent. However, the possibility remains that the glyoxylate aminotransferase activity observed in the chloroplast preparations was largely of peroxisomal origin. To examine this point the chloroplast preparations were routinely examined for catalase activity as high levels of this enzyme occur in peroxisomes in contrast to the low levels present in chloroplasts.[37] Using a sensitive spectrophotometric assay[39] only extremely low levels of this enzyme could be detected in non-aqueously isolated pea chloroplasts. Thus it appears that contamination of the chloroplasts by peroxisomal protein was very small and it is logical to conclude that the observed synthesis of glycine and serine by these preparations was largely catalyzed by enzymes of chloroplastic origin.

Serine and glycine produced within the chloroplast would conceivably be derived from photosynthetic intermediates. There are reports in the literature that illuminated chloroplasts can incorporate bicarbonate into these amino acids. For example, Chang and Tolbert[40] reported that spinach chloroplasts synthesized uniformly labelled glycine from [^{14}C]-bicarbonate together with serine labelled predominantly in the carboxyl carbon. Work in Bassham's laboratory[41,42] has also provided evidence for incorporation of $^{14}CO_2$ into glycine and furthermore has demonstrated that glycollate is converted into glyoxylate and glycine by isolated chloroplasts. Besides being utilized for terpenoid biosynthesis it is possible that serine, synthesized within the chloroplast, could play an important role in one-carbon metabolism. The presence of both formyl and methyl derivatives of H_4PteGlu in chloroplasts[25,26] suggests that oxidation and reduction of one-carbon units can occur in this organelle. These possibilities are currently under investigation and will be the subject of a later publication.

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EXPERIMENTAL

Chemicals

[^{14}C]-Formaldehyde, [2-^{14}C]-glyoxylate, [2-^{14}C]-glycine and L-[3-^{14}C]-serine were purchased from Amersham-Searle Corporation, Des Plaines, Illinois, U.S.A. Labelled glyoxylate from the supplier contained small amounts of an impurity which was removed by TLC using the solvent system described below. H_4PteGlu was purchased from Nutritional Biochemicals Corporation, Cleveland, U.S.A., and $H_4PteGlu_3$ was prepared by reduction of $PteGlu_3$.[43] The purity of these preparations was greater than 95 per cent as determined by chromatography on DEAE-cellulose.[44] The concentrations of H_4PteGlu and $H_4PteGlu_3$ were measured by microbiological assay[45] using *Lactobacillus casei* (ATCC 7469).

Plant Material

Pea (*Pisum sativum* var. Homesteader) plants were grown in moist vermiculite in a greenhouse for 15 days, and then placed in darkness for 24 hr in order to deplete the starch content. Young developing leaves from the seedlings were then carefully selected, deveined and immediately frozen and lyophilized.

Chloroplast Preparation

Chloroplasts were isolated non-aqueously by density gradient centrifugation using *n*-hexane/CCl_4. The procedure used was a modification of the method previously described by Rogers *et al.*[46] The density gradients used had specific gravities of 1·34, 1·32 and 1·30. The final chloroplast pellet obtained, after washing three times with *n*-hexane and drying *in vacuo* at −4°, was suspended in 1·5 ml of 0·15 M potassium phosphate buffer (pH 7·4) and subjected to ultrasonic treatment[46] immediately before use.

Assay of Glyoxylate Aminotransferase Activity

Glyoxylate aminotransferase activity in chloroplast preparations was measured by ability to form [2-^{14}C]-glycine from [2-^{14}C]-glyoxylate in the presence of various amino group donors. For the assay, each reaction tube contained [2-^{14}C]-glyoxylate, pyridoxal-5′-phosphate, $MgSO_4$, K-phosphate buffer (pH 7·4), L-amino acid and chloroplast extract as shown in Table 1. Controls contained all of the above components with the exception that the chloroplast preparation was boiled for 2 min prior to use.

The reaction was terminated by adding 1·5 ml boiling 80 per cent (v/v) aq. ethanol. After removal of precipitated protein by centrifugation, the contents of each tube were fractionated by chromatography on Dowex 50W X8 (H^+ form) columns.[47] The organic acid fraction was eluted with water and discarded. The column was then washed with 2 N NH_4OH to obtain the amino acids. This fraction was neutralized and the total volume reduced *in vacuo* to facilitate chromatography on thin-layer plates coated with cellulose MN300. Chromatography was carried out using *n*-butanol:acetone:water:diethylamine (20:18:10:3, by vol.); good separation of glycine and serine was achieved, the R_f's being 0·35 and 0·55 respectively. Labelled glycine and serine were located on the plates by autoradiography. The radioactive areas were then removed after treatment of the plates with pyroxylin.[48] Radioactivity was measured in a Nuclear Chicago Liquid scintillation counter (Model Unilux II) using a scintillation fluid containing 6 g of 2,5-diphenyloxazole and 0·4 g of 1,4-*bis* 2-(4-methyl-5-phenyloxazolyl)benzene per litre of toluene.

Synthesis of Serine from Glycine by Chloroplast Extracts

The conversion of glycine to serine was measured in reaction systems containing chloroplast extracts, H_4PteGlu, pyridoxal-5′-phosphate, [2-^{14}C]-glycine and K-phosphate buffer (pH 7·4) as shown in Table 2. The incubation was carried out at 30° and terminated by addition of boiling 80 per cent (v/v) aqueous ethanol after 30 min. The contents of each tube were fractionated and the amino acids assayed for radioactivity as described for glyoxylate aminotransferase. In complementary assay systems [2-^{14}C]-glycine was replaced by [^{14}C]-formaldehyde.

Assay of Serine Hydroxymethyltransferase Activity

Serine hydroxymethyltransferase activity was assayed by the method of Taylor and Weissbach.[49] The reaction system, in a final volume of 1·0 ml, contained 60·0 μM K-phosphate buffer (pH 7·4); 0·12 μM L-[3-^{14}C]-serine (8·5 μc/μM); 8 μM pyridoxal-5′-phosphate; 2·5 μM H_4PteGlu or 2·5 μM $H_4PteGlu_3$; 6·0 μM

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2-mercapto-ethanol and chloroplast extract containing 1–2 mg protein. Incubation was carried out for 20 min at 30° and radioactivity in the final formaldehyde dimedone derivative determined by liquid scintillation counting.

Assay of Protein

Total protein in chloroplast extracts was determined colorimetrically[50] after prior removal of pigments with hot acid-ethanol.[51] Crystalline bovine serum albumin was used as the standard.

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