PHOSPHOSERINE AS AN EARLY PRODUCT OF PHOTOSYNTHESIS IN ISOLATED CHLOROPLASTS AND IN LEAVES OF *ZEA MAYS* SEEDLINGS

David J. CHAPMAN and Rachel M. LEECH
Department of Biology, University of York, York YO1 5DD, England

Received 9 July 1976

1. Introduction

Recent studies of metabolite exchange in photosynthesising tissues and in isolated chloroplasts clearly indicate that newly assimilated carbon is rapidly exported out of the chloroplast as triosephosphate [1,2]. Since triosephosphate can be subsequently converted to 3-PGA in the cytosol [3], dihydroxyacetone phosphate can provide a major source of photosynthetically produced reducing and phosphorylating equivalents to the rest of the cell [1,2]. In the light of such findings it seems pertinent to examine further possible roles of the chloroplast in the provision of other photoassimilated metabolites to the cytoplasm. Some amino acids are also formed during cellular photosynthesis by the amination of photoassimilated carbon compounds. The production of reduced nitrogen needed for this amination also appears to be a major chloroplast function since recent evidence suggests that enzymes for the reduction of nitrate and nitrite to ammonia, the incorporation of ammonia into glutamate and glutamine, and also several aminotransferases are localised within chloroplasts from leaf cells. (For review, see [4].) A quantitative comparison of the range of amino acids produced from $^{14}CO_2$ in leaf tissue and also in isolated chloroplast during short term photosynthesis would indicate which of the amino acids are synthesised within the chloroplasts, using photoassimilated carbon skeletons.

The use of the improved techniques of automatic amino acid analysis for the routine identification and measurement of amino acids and ^{14}C radioactivity levels now makes possible the quantitative analysis of all the free amino acids which became labelled during short periods of photoassimilation of $^{14}CO_2$.

This paper reports the discovery that phosphoserine is a major amino acid produced in photosynthesising chloroplasts and suggests the possibility that this compound represents a phosphorylated and aminated carbon source to the cytoplasm.

2. Materials and methods

Seedlings of *Zea mays* (var. Kelvedon Glory) were grown as described previously [5]. *Zea mays* was chosen since the proportion of assimilated carbon moving into amino acids is high in this plant and also critical enzyme location studies have been performed with maize and other C_4 species [6–8].

Intact seedlings (in batches of 6 in one pot) were exposed to $^{14}CO_2$ of known specific activity for periods of 20 sec or 120 sec. In the 'pulse chase' experiments exposure to $^{14}CO_2$ for 20 sec was followed by a period of 100 sec in which $^{14}CO_2$ was displaced by $^{12}CO_2$. The procedure used for exposure of seedlings gave little disturbance in the plant environment; thus the 'steady-state' situation was not interrupted and the total CO_2 ($^{12}CO_2 + {}^{14}CO_2$) concentration was constant throughout the exposure [9]. The assimilation period was ended by plunging the leaf material into liquid nitrogen.

Chloroplasts were isolated from expanded leaf tissue 6 cm from the shoot base of the maize seedlings. The isolation procedure of Leese and Leech [10] was modified to reduce the severity of the leaf homo-

 North-Holland Publishing Company – Amsterdam

genisation, by using two consecutive periods of 3 sec and 5 sec at full speed in an MSE atomix. The isolation medium of O'Neal and coworkers [11] was used with an increased sorbitol concentration of 0.5 M.

The chloroplast suspensions (between 0.15 and 0.25 mg chlorophyll/ml) contained up to 63% intact chloroplasts in different experiments, as indicated by phase contrast microscopy. The ratio of intact mesophyll:intact bundle sheath chloroplasts in the suspensions was found to be approx. 10:1 by electron microscopy. Chloroplast incubations were terminated after 12 min by addition of 5 N formic acid, the soluble compounds were extracted, and the amino acids separated by cation exchange resin and individual components were analysed as for the leaf extracts.

3. Results and discussion

Exposure of leaves attached to intact maize seedlings to $^{14}CO_2$ for a period of 20 sec resulted in the radioactive labelling of some free amino acids. The assimilation of ^{14}C into individual amino acid pools of a specific area of the maize seedling leaf is shown in table 1. As expected from the operation of the C_4 pathway of photosynthesis the four carbon com-

Table 1
^{14}Carbon assimilation into amino acid pools of intact leaf tissue

	Relative specific activity %		
	A 20 sec $^{14}CO_2$	B 120 sec $^{14}CO_2$	C 20 sec $^{14}CO_2$ plus 100 sec $^{12}CO_2$
Aspartate	5.1	10.9	5.5
Phosphoserine	0.3	1.2	0.6
Alaine	0	1.2	0.5
Glutamate	0	0.5	0.1
Glycine	0	0.5	0.2
Serine	0	0.3	0.2

The relative specific activities of the six most heavily labelled amino acids in the short section 8 to 10 cm. from the base was determined after continuous exposure to $^{14}CO_2$ and after a 20 sec $^{14}CO_2$ 'pulse' followed by a 100 sec $^{12}CO_2$ 'chase'. Labelled metabolites were extracted in 80% ethanol and pigments removed from the extract by thin-layer chromatography on Silica Gel H in a chloroform : methanol : water (65:25:45 v/v) solvent system. Plate material below the 0.5 R_F position was removed and washed with water and the combined washings mixed with a cation exchange resin to separate the amino acid fraction [12]. Amino acids were identified and cold pool sizes measured using a JEOL fully automatic amino acid analyser, model JLC-6AH. Radioactivity in amino acids was measured by passing a proportion of the eluate from the analyser column through a Nuclear Chicago continuous flow scintillation detector. The relative specific activity was calculated as a percentage by:

$$\frac{\text{Specific activity in amino acid} \times 100}{\text{Specific activity in } ^{14}CO_2 \text{ at beginning of exposure}}$$

where specific activity is calculated by dividing radioactivity (nCi) in compound by nmoles of compound. Figures in A are averages from two experiments, figures in B and C are from one experiment each. Specific activities of $^{14}CO_2$ at the beginning of the exposures were: A, 46 and 44 nCi/nmol; B, 47 nCi/nmol, and C, 36 nCi/nmol.

pound, aspartic acid, was the most heavily labelled amino acid containing more than 97% of the total ^{14}C radioactivity in the amino acid fraction, in two 20 sec experiments. Of the amino acids other than aspartic acid, phosphoserine contained the most ^{14}C radioactivity, i.e. between 1.4% and 2.0% of the total activity in amino acids. In the analysis of six separate batches of leaf material ^{14}C in phosphoserine was always a similar proportion of the ^{14}C amino acid fraction, i.e. between 2.0% and 2.5%. Low levels of radioactivity were recorded in alanine, glycine, serine and glutamic acid, all less than 0.4% of the total ^{14}C in the amino acid fraction.

The rate of assimilation into phosphoserine was almost the same in 20 sec and 120 sec exposures but the rate of assimilation into other amino acids, with the exception of aspartic acid, was much greater in the 120 sec assimilation period than in the 20 sec period. The rapid incorporation in the first 20 sec indicates that there is a more direct assimilation of CO_2 into phosphoserine than into the amino acids alanine, glycine, serine and glutamic acid. However, the total phosphoserine pool in this tissue contained more ^{14}C radioactivity after the experiment in which seedlings were exposed to a $^{14}CO_2$ 'pulse' of 20 sec followed by a period of 100 sec 'chase' by $^{12}CO_2$, than in the experiments where $^{14}CO_2$ was supplied for 20 sec. This suggests the possibility that some of the phosphoserine may not be assimilated directly from CO_2.

When chloroplasts were isolated from maize seedling leaf tissue by an aqueous method (see Methods) and incubated with NaH $^{14}CO_3$ only three amino acids were found to be labelled. These were phosphoserine (between 55% and 61%) and aspartic acid (between 38% and 45%), together accounting for at least 98% of the total amino acid radioactivity, with glycine only detected at a very low level (table 2). In the analysis of ^{14}C assimilation in 10 different incubations of chloroplasts isolated on 3 different occasions no other radioactive amino acids were found.

In contrast to the labelling pattern for intact leaves, in the isolated chloroplasts that fixed CO_2 at rates greater than 7.0 μmol/mg chlorophyll/h, phosphoserine contained more activity than aspartic acid. In 3 separate experiments the ratio of activities of phosphoserine:aspartic acid varied between 1.6 and 1.0. Rates of ^{14}C assimilation by the isolated chloroplasts

Table 2
Distribution of ^{14}Carbon in amino acids after incubation of isolated chloroplasts with $H^{14}CO_3^-$

	A ^{14}C incorporation	B nCi/mg chl/12 min
Phosphoserine	183	338
Aspartate	148	214
Glycine	0	7
*Other amino acids and amides	0	0

Isolated chloroplasts were incubated with NaH $^{14}CO_3$ for 12 min at 25°C with 10 mM ribose 5-phosphate in experiments A and B, and 2 mM Phosphoenolpyruvate in experiment A but 4 mM Phosphoenolpyruvate in experiment B. The incubation medium contained 0.5 M sorbitol, 5.0% (w/v) PVP, 0.05 M HEPES buffer at pH 7.8, 1.0 mM $MgCl_2$, 1.0 mM EDTA, 0.2 M KH_2PO_4, 5.0 mM dithiothreitol, 40 mM sodium isoascorbate and a total concentration of 10 mM $NaHCO_3$ at a specific radioactivity of between 2.0 and 5.0 nCi/nmol. Acid stable products were separated and amino acids analysed as described in the methods section. The contents of two incubation vials were combined for each analysis. Radioactivity recorded as 1 nCi was equivalent to about 620 cpm in the detection system.

*More than 24 different other amino acids and 2 amides were identified in extracts of the incubated chloroplasts and were not found to be radioactive.

of up to 27 μmol/mg chlorophyll/h were recorded. (This is equivalent to 42 μmoles per hour when the rate is corrected to account for the lack of carbon assimilation by the broken chloroplasts present in the suspension [13].) Uncorrected rates of between 7 and 27 μmol CO_2/mg chlorophyll/h were recorded for 5 different batches of chloroplasts. The amino acid fraction represented between 10 and 17% of the total ^{14}C radioactivity assimilated in 12 min incubations. Phosphoserine was found to be both an early radioactive product of $^{14}CO_2$ assimilation in intact leaf tissue and the most abundant radioactive amino acid product of $H^{14}CO_3^-$ assimilation by an isolated chloroplast suspension. Investigations of the radioactive products of $^{14}CO_2$ assimilation reported by other workers have not detected radioactivity in phosphoserine, possibly because in most solvents used to separate photosynthetic intermediates, phosphoserine remains close to the origin.

In the present studies, 70% of the chloroplast phosphoserine pool became labelled during the first twelve minutes of CO_2 fixation in isolated chloroplasts (assuming only the carboxyl carbon is initially labelled). Rates of synthesis of phosphoserine of approx. 1 μmol/mg chlorophyll/h were recorded and while it is unlikely that optimal conditions have been established, the rates compare favourably with rates of 6–12 μmol/mg chlorophyl/h for the photoreduction of nitrite to all amino compounds in isolated chloroplasts, reported by Magalhaes et al. [14] for spinach chloroplasts, fixing $^{14}CO_2$ at 100 μmol/mg chlorophyll/h.

Chloroplasts show limited permeability to non-phosphorylated amino acids [1,15] and of those produced during photosynthesis only aspartate and glutamate have been shown to cross the chloroplast envelope at rates higher than about 1.0 μmol/mg chlorophyll/h by a specific translocase [1,16]. The light-dependent incorporation of $^{14}CO_2$ into a phosphorylated amino acid in chloroplasts is therefore of considerable interest and the characteristics of the phosphoserine molecule suggest it may be rapidly translocated across the chloroplast envelope membrane. Direct experimental evidence is required to demonstrate whether the phosphate translocator of the inner chloroplast envelope membrane [1,16] can facilitate the transport of this phosphorylated aminated product of photosynthesis into the cytosol or whether other mechanisms are involved.

In non-green plant tissue the pathway of synthesis of phosphoserine appears to be from 3-phosphoglycerate via phosphohydroxypyruvate [17] and a similar two enzyme reaction from photosynthetically synthesised 3-PGA could operate in the chloroplast [18]. D-3-Phosphoglycerate dehydrogenase, the enzyme catalysing the conversion of 3-phosphoglycerate to phosphohydroxypyruvate has been shown to be present in leaves [19], although neither this activity nor the conversion of phosphohydroxypyruvate to phosphoserine has been examined in chloroplasts.

After this work was complete, the synthesis of phosphoserine was an early product of photosynthesis in leaves of *Phaseolus vulgaris*, a C_3 plant, was reported [20] showing that the photosynthesis of phosphoserine is not restricted to plants showing the C_4 pattern of carbon fixation.

References

[1] Heber, U. (1974) Ann. Rev. Plant Physiol. 251, 393–421.
[2] Walker, D. A. (1974) in: M.T.P. International Review of Science. Biochemistry Section (D. H. Northcote, ed.), Vol. 11, pp. 1–49, Butterworths, London.
[3] Stocking, C. R. and Larson, S. (1969) Biochem. Biophys. Res. Commun. 37, 278–282.
[4] Leech, R. M. and Murphy, D. J. (1976) in: The Intact Chloroplast (J. Barber, ed.), pp. 365–401, Elsevier Amsterdam.
[5] Leech, R. M., Rumsby, M. G. and Thomson, W. W. (1973) Plant Physiol. 52, 240–245.
[6] Mellor, G. E. and Tregunna, E. B. (1971) Can. J. Bot. 49, 137–142.
[7] Rathnam, C. K. M. and Das, V. S. R. (1974) Can. J. Bot. 52, 2599–2605.
[8] Slack, C. R., Hatch, M. D. and Goodchild, D. J. (1969) Biochem. J. 114, 489–498.
[9] Chapman, D. J. (1976) D. Phil. thesis. University of York, UK.
[10] Leese, B. M., Leech, R. M. and Thomson, W. W. (1972) 2nd International Congress Photosynthesis Research, 2, 1485–1494, Stresa, Italy.
[11] O'Neal, D., Hew, C. S., Latzko, E. and Gibbs, M. (1972) Plant Physiol. 49, 607–614.
[12] Kirk, P. R. and Leech, R. M. (1972) Plant Physiol. 50, 228–234.
[13] Lilley, R. McC. and Walker, D. A. (1975) Plant Physiol. 55, 1087–1092.
[14] Magalhaes, A. C., Neyra, C. A. and Hageman, R. H. (1974) Plant Physiol. 53, 411–415.

[15] Gimmler, H., Schäfer, G., Kraminer, H. and Heber, U. (1974) Planta, 120, 47–61.
[16] Heldt, H. W., Fliege, R., Lehner, K., Milovancev, M. and Werdan, K. (1975) in: Proceedings of 3rd International Congress on Photosynthesis, Rehovot. (M. Avron, ed.) 2, pp. 1369–1379, Elsevier, Amsterdam.
[17] Hanford, J. and Davies, D. D. (1958) Nature 182, 532–533.
[18] Chang, W. H. and Tolbert, N. E. (1965) Plant Physiol. 40, 1048–1052.
[19] Cheung, G. P., Rosenblum, I. Y. and Sallach, H. J. (1968) Plant Physiol. 43, 1813–1820.
[20] Daley, L. S. and Bidwell, R. G. S. (1976) Plant Physiol. Suppl. 57, 5.