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PHOTOSYNTHETIC $^{14}CO_2$ FIXATION BY CHLOROPLAST POPULATIONS ISOLATED BY A POLYMER TWO-PHASE TECHNIQUE

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SUMMARY

Preparations of spinach chloroplasts, essentially free from contamination by other cellular material or whole cells, incorporated ^{14}C almost entirely into glycolate, a polyglucan (probably starch) and intermediates of the Calvin cycle and starch synthesis. About 70 % of the ^{14}C was found in dihydroxyacetone phosphate, 3-phosphoglycerate and glycolate. Only small amounts were found in sucrose and amino acids.

On the other hand, preparations consisting of particles containing chloroplasts surrounded by a membrane-bound cytoplasmic layer including mitochondria and microbodies, gave a much broader spectrum of ^{14}C-labelled products. Much less of the ^{14}C was found in dihydroxyacetone phosphate and 3-phosphoglycerate. Instead, sucrose, malate, aspartate, alanine and some other amino acids contained about 40 % of the ^{14}C incorporated. It is concluded that sucrose is synthesized by cooperation between the chloroplast and the surrounding layer.

INTRODUCTION

Chloroplasts from spinach can be separated into three populations by countercurrent distribution, using a dextran–polyethylene glycol two-phase system [1, 2]. The three cholorplast populations differ in morphology [2], protein/chlorophyll ratio [2] and enzyme content (unpublished observations). One population, Peak I, consists of intact chloroplasts surrounded by the chloroplast envelope, so-called Class I chloroplasts. The second population, Peak II, consists of chloroplasts which have lost their envelopes and much of their stromal material, so-called Class II chloroplasts. Finally the third population, Peak III, consists of particles containing intact cholorplasts surrounded by a membrane-bound cytoplasmic layer including mitochondria and microbodies.

We now report the $^{14}CO_2$ fixation capacity of these chloroplast populations and also the patterns of incorporation of ^{14}C into different metabolites. To this end we have developed a more rapid method than that previously used to isolate the chloroplast populations. This involves rapid centrifugation followed by a few phase partition steps.

The results show that both Peak I and Peak III chloroplasts have a good $^{14}CO_2$ fixation capacity. Their incorporation patterns, however, are very different. The Peak III chloroplasts have an incorporation pattern which is more like that of intact leaf tissue. It is also concluded that sucrose is synthesized by cooperation between the chloroplast and the surrounding layer.

MATERIALS AND METHODS

Preparation of Peak I (Class I) chloroplasts

Chloroplasts were prepared essentially according to Kalberer et al. [3] from 20 g of full-grown leaves or from 30 g of cotyledons. This preparation in the form of a well-drained pellet containing about 50 % Class I chloroplasts as judged by phase-contrast microscopy was suspended in 5 ml top-phase+3 ml bottom-phase of phase-system A, described earlier [2] (except that the phase-system contained 0.30 M sorbitol instead of 0.22 M sucrose). The phase-system was mixed and centrifuged for 1.5 min at 2400 rev./min ($900 \times g$ at bottom of the centrifuge tube) in a swing-out centrifuge (Christ) to obtain phase separation. The top-phase, containing mainly broken chloroplasts and contaminants was removed and discarded. The bottom-phase was washed once with 5 ml fresh top-phase diluted with 20 ml of the preparative solution [3] and the chloroplasts were spun down at $500 \times g$ for 5 min. The pellet, containing 70–90 % Class I chloroplasts, was resuspended in assay solution according to Kalberer et al. [3] and used in the $^{14}CO_2$ fixation experiments.

Preparation of Peak III chloroplasts

This chloroplast population was always prepared from cotyledons, using the step-wise grinding procedure described earlier [2]. Otherwise, the initial preparation was in this case essentially according to Kalberer et al. [2]. 30 g of cotyledons were used. The well-drained chloroplast pellets from the last two of three successive grindings were suspended in 10 ml top-phase +10 ml bottom-phase of phase-system B, described earlier [2] (except that the phase-system contained 0.30 M sorbitol instead of 0.22 M sucrose). The phase-system was transferred to a small separatory funnel and left to separate. After a separation time of 15 min, the bottom-phase was carefully removed and discarded. The top-phase was washed once with 10 ml fresh bottom-phase, diluted with 10 ml of the preparative solution [3] and the Peak III chloroplasts were collected by centrifugation at $500 \times g$ for 5 min. Chlorophyll was determined by the method of Arnon [4].

$^{14}CO_2$ fixation by chloroplast suspensions

0.5 ml chloroplast suspension (15–100 μg chlorophyll) was transferred to a round-bottomed transparent tube, 25 mm in diameter. The tube was kept in a waterbath (21 °C) in slow rotation, to make the suspension swirl round the bottom of the tube. The suspension was illuminated under air from two sides by two 150 W lamps (Philips, Comptalux) with a total intensity of 80000 lux. After 3 min pre-illumination, 20 μl of 34 mM $KH^{14}CO_3$ (59 Ci/mole) or 20 μl of 167 mM $KH^{14}CO_3$ (6 Ci/mole) was added to give an $H^{14}CO_3^-$ concentration of 1.3 mM or 6.4 mM, respectively. After another 6 min, the incorporation was terminated by the addition of 0.25 ml 1 M HCl.

$^{14}CO_2$ fixation by leaf pieces

The procedure was the same as for a chloroplast suspension, except for the material: about 20 leaf pieces (2mm × 2 mm) infiltrated under reduced pressure with 0.5 ml of the assay solution. Extraction of soluble material from the leaf pieces was facilitated by freeze-thawing.

To determine the total incorporation of ^{14}C, 10 μl of the acidified extract were spotted on a small filter paper. The filter paper was dried at 70 °C and the non-volatile radioactivity measured by liquid scintillation counting at 55 % efficiency.

Separation, identification and radioactivity counting of the products of $^{14}CO_2$ fixation were essentially according to Schürmann et al. [5, 6]. Thus the soluble ^{14}C-labelled products were identified by co-electrophoresis and/or co-chromatography with the authentic compounds, except for dihydroxyacetone phosphate and glycerate, which were identified by the schedule of Schürmann [5]. All the "co-runs" done by us were found to coincide with the schedule of Schürmann [5].

Insoluble material was pelleted by centrifugation and hydrolyzed for 6 h in 6 M HCl at 80 °C. After that treatment, most of the radioactivity from both Peaks I and III insolubles co-chromatographed with glucose. ^{14}C-labelled insoluble products are therefore referred to as starch.

RESULTS AND DISCUSSION

Both Peak I and Peak III chloroplasts fixed $^{14}CO_2$ at an average rate of 30–60 μmoles/mg chlorophyll per h over the first 6 min with 6.4 mM HCO_3^- and at about half that rate with 1.3 mM HCO_3^- as starting concentration. The $^{14}CO_2$ fixation of Peak II (Class II) chloroplasts was, as expected, negligible. The pattern of incorporation of ^{14}C into different metabolites after 6 min photosynthesis with 1.3 mM HCO_3^- as starting concentration was investigated for Peak I and Peak III chloroplasts and for leaf pieces.

Peak I chloroplasts, i.e. Class I chloroplasts essentially free from contamination by other cellular material or whole cells, incorporated ^{14}C almost entirely into glycolate, a polyglucan (starch) and intermediates of the Calvin cycle and starch synthesis. About 70 % of the incorporation was found in dihydroxyacetone phosphate, 3-phosphoglycerate and glycolate, only small amounts in sucrose and amino acids (Table I, Fig. 1). No qualitative difference in incorporation pattern was found between Peak I chloroplasts prepared from cotyledons and full-grown leaves. This incorporation pattern agrees well with those obtained by other investigators with similar preparations [6–10]. The high amounts of radioactivity found in dihydroxyacetone phosphate, 3-phosphoglycerate and glycolate after photosynthetic $^{14}CO_2$ fixation with Class I chloroplasts in suspension have been explained by the fact that these compounds readily leak through the chloroplast envelopes [8, 11] and accumulate in the medium [8].

On the other hand, Peak III preparations (consisting of particles containing one, and sometimes two or three chloroplasts surrounded by a membrane-bound cytoplasmic layer including mitochondria and microbodies [2]) gave a much broader spectrum of ^{14}C-labelled products. Much less of the ^{14}C was found in dihydroxyacetone phosphate and 3-phosphoglycerate. Instead, sucrose, malate, aspartate, alanine and some other amino acids contained about 40 % of the ^{14}C incorporated (Table I,

TABLE I

PATTERNS OF INCORPORATION OF ^{14}C INTO DIFFERENT METABOLITES AFTER 6 min PHOTOSYNTHETIC $^{14}CO_2$ FIXATION AS PERCENTAGE OF TOTAL RADIOACTIVITY

	Peak I chloroplasts		Peak III chloroplasts			Leaf pieces	
	Cotyledons	Full grown leaves	Expt 1	Expt 2	Expt 3	Cotyledons	Full grown leaves
Sucrose	0.7	0.2	6.9	6.1	16.5*	4.7	7.4
Sugar diphosphates	4.0	5.2	6.3	8.1	9.8	1.3	3.7
Sugar monophosphates	20.6	11.5	15.8	24.4	17.4	14.2	9.7
Dihydroxyacetone phosphate	20.3	48.6	5.2	3.6	4.1	12.7	2.3
3-Phosphoglycerate	43.0	22.0	3.5**	7.6**	7.8**	6.9**	13.0**
Phosphoenolpyruvate	–	0.2	0.3	0.3	0.3	0.4	0.7
Phosphoglycolate	0.1	0.1	–	–	–	–	–
Aspartate	–	–	2.6	4.6	5.1	4.9	6.9
Glutamate	–	–	0.4	0.4	0.4	1.2	1.1
Alanine	1.4	–	4.5	4.1	7.5	1.9	6.0
Serine		2.5	4.5	3.4	8.4	8.8	13.2
Glycine			4.9	1.2		3.3	3.9
Threonine		–	0.4	0.3		0.5	–
Asparagine		–	11.6	6.9	(10.0)*	7.5	3.4
Total amino acids	1.4	2.5	28.9	20.9	(31.4)*	28.1	34.5
Glycolate	4.0	5.3	4.7	2.0	0.8	3.9	0.8
Malate	–	–	8.4	9.9	7.9	16.1	14.8
Glycerate	–	–	3.4	2.0	0.7	2.2	3.5
Starch	1.4	2.7	11.4	10.0***	9.7	4.4	7.2
Unidentified compounds	4.3	1.6	5.2	5.2	3.6	5.1	2.2
μmoles $^{14}CO_2$/mg chlorophyll per h	32	14	13	24	28	66	–

* No separation was achieved between sucrose and asparagine. Assuming 6.5% sucrose gives the figures inside brackets for asparagine and total amino acids in Expt. 3.

** Co-electrophoresis and co-chromatography with 2-phosphoglycerate and Peak III chloroplast extract indicated that about half of the 3-phosphoglycerate spot was indeed 2-phosphoglycerate. This was not done with leaf piece extract, but it seems reasonable that the corresponding spot from leaf piece extract was also a mixture of 2- and 3-phosphoglycerate.

*** The incorporation of ^{14}C into insolubles was not measured in Expt 2. All figures in this experiment are calculated assuming that 10% of total was incorporated into insolubles.

Fig. 2). Further, a co-run with 2-phosphoglycerate indicated that about half the 3-phosphoglycerate spot was indeed 2-phosphoglycerate, the pool of which was probably present in the surrounding layer. This pattern of incorporation is more like that of intact cotyledon or full grown leaf tissue (Table I). Obviously, ^{14}C-labelled products initially formed in the chloroplast were exported to the surrounding layer and further metabolized. Probably there was also a contribution from β-carboxylation of phosphoenolpyruvate in the surrounding layer. Furthermore, there was also a higher

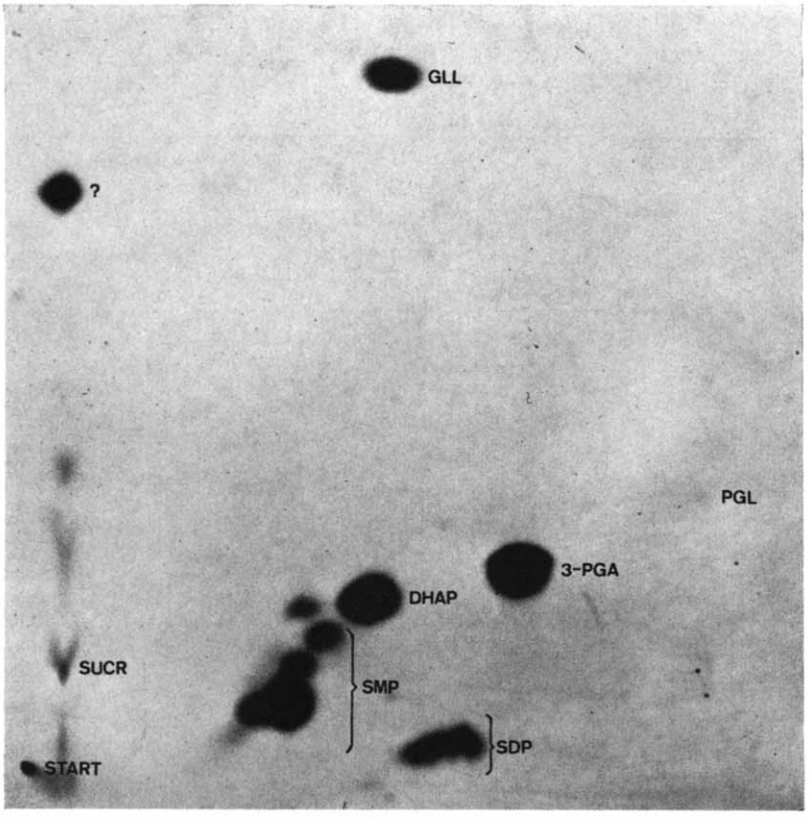

Fig. 1. Radioautograph of ^{14}C-labelled products after 6 min photosynthetic $^{14}CO_2$ fixation by a Peak I chloroplast preparation from cotyledons (the same as shown in Table I). Products were separated by the thin-layer electrophoresis (horizontal)–chromatography (vertical) technique, according to Schürmann [5]. This chloroplast preparation, consisting mainly of intact chloroplasts, was essentially free from contamination by other cellular material or whole cells. Abbreviations: GLL, glycolate; MAL, malate; GLR, glycerate; PEP, phosphoenolpyruvate; PGL, phosphoglycolate; 3-PGA, 3-phosphoglycerate; DHAP, dihydroxyacetone phosphate; SMP, sugar monophosphates; SDP, sugar diphosphates; ASP, aspartate; GLU, glutamate; ALA, alanine; THR, threonine; GLY, glycine; SER, serine; ASN, asparagine; SUCR, sucrose.

incorporation of ^{14}C into starch (Table I).

The results for Peak III chloroplasts agree reasonably well with those obtained by Bidwell et al. [12] with *Acetabularia* chloroplasts, which are also surrounded by a layer of extrachloroplastic material [12]. However, $^{14}CO_2$ fixation by *Acetabularia* chloroplasts free from contamination of other cellular material has not been described. Therefore the role of the surrounding layer in $^{14}CO_2$ fixation with *Acetabularia* chloroplasts is not known [13].

If our results reflect the situation in situ, the ability of the chloroplasts themselves to synthesize compounds from assimilated CO_2 would seem to be rather limited. Two of their main roles would be accumulation of sugar as starch and the export of dihydroxyacetone phosphate, 3-phosphoglycerate and glycolate to the rest of the cell, for the further synthesis of sucrose, amino acids and other compounds,

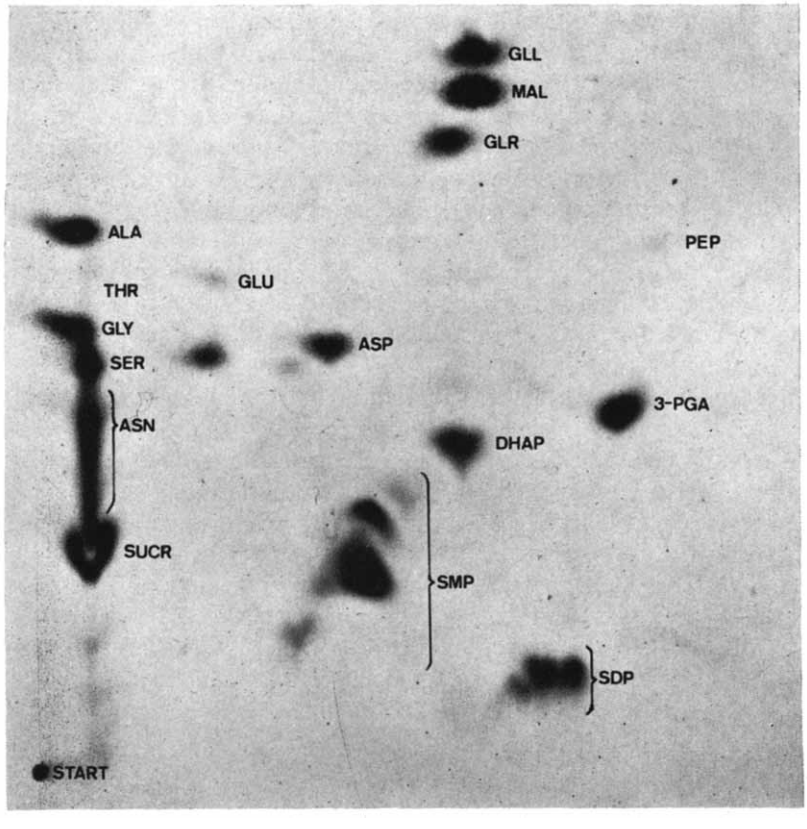

Fig. 2. Radioautograph of ^{14}C-labelled products after 6 min photosynthetic $^{14}CO_2$ fixation by a Peak III chloroplast preparation from cotyledons (the same as Expt 2 in Table I). Products were separated by thin-layer elctrophoresis (horizontal) – chromatography (vertical) technique, according to Schürmann [5]. This chloroplast preparation, consisting of particles containing chloroplasts surrounded by a membrane-bound cytoplasmic layer including mitochondria and microbodies, gave a pattern of incorporation quite similar to that of intact leaf tissue (Table I), but very different from that of Peak I (Class I) chloroplasts (Compare Fig. 1 and Table I). Abbreviations: see Fig. 1.

as discussed by Grant et al. [9]. And, the chloroplasts themselves would be dependent on the rest of the cell for many compounds, as has been shown for the synthesis of some amino acids [14, 15].

A high incorporation of ^{14}C into sucrose by isolated chloroplasts has only rarely been reported [12, 13, 16]. Although Coombs and Baldry [10] minimized the leakage of dihydroxyacetone phosphate and 3-phosphoglycerate by illuminating the chloroplasts on a filter paper support, and thus could make the preparations produce mainly hexose monophosphates, they could not elicit the production of sucrose. They therefore suggested that sucrose is synthesized, not in the chloroplasts but in the cytoplasm, from an intermediate originating from the chloroplasts [10].

Our Peak I chloroplast preparations consist mainly of Class I chloroplasts with only minor amounts of non-chloroplastic contaminants. These chloroplasts are effective in $^{14}CO_2$ fixation, but they do not synthesize sucrose. In contrast, Peak III

chloroplasts having a layer of extrachloroplastic material are very effective in the synthesis of sucrose. We therefore conclude that the capacity to synthesize sucrose is due to cooperation between the chloroplast proper and the surrounding layer. Since the chloroplast can store sugar as starch, it seems more reasonable that biosynthesis of sucrose is the way to store and translocate sugar outside the chloroplast, preferably by synthesizing it in the cytoplasm from the compounds known to leak from the chloroplasts (dihydroxyacetone phosphate, 3-phosphoglycerate and glycolate).

That part of the synthesis of sucrose occurs outside the chloroplast is supported by recent studies on the localization of UDPglucose–fructosephosphate glycosyltransferase and UDPglucose pyrophosphorylase by Bird et al. [17]. Most of these enzymes were found in the supernatant and very little in the chloroplast fraction [17].

The above would also mean that the amounts of ^{14}C found in sucrose, amino acids and some other compounds after photosynthetic $^{14}CO_2$ fixation with Class I chloroplasts merely reflect the degree of contamination by other cellular material.

Peak I and Peak III preparations offer a unique opportunity to study the cooperation between the chloroplast and its natural environment. By studying "difference metabolism", that is comparing Peak I and III under identical conditions, we can learn about the metabolism of the cytoplasmic layer, the flow of metabolites out from and into the chloroplast and so forth. Generally studies on such higher structures composed of more than one type of organelle (chloroplasts, mitochondria, peroxisomes) embedded in the cytoplasmic "background" should increase our understanding of the localization, integration and control of cell metabolism.

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