THE SITE OF SUCROSE SYNTHESIS IN ISOLATED LEAF PROTOPLASTS

S. P. ROBINSON and D. A. WALKER
Botany Department, The University, Sheffield, S10 2TN, England

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1. Introduction

Sucrose is one of the major products of photosynthesis and the principal metabolite exported from leaves, yet its site of synthesis in the cell has been a matter of debate for some time [1]. Nonaqueous fractionation of leaf tissue [2] suggested that at least some of the enzymes responsible for sucrose synthesis were located in the chloroplast but the nonaqueous chloroplasts were contaminated by cytoplasm [3]. Later work [4] showed that only a small percentage of the activity of these enzymes was recovered in chloroplasts prepared by aqueous methods suggesting a cytoplasmic site for sucrose synthesis. Studies on the distribution of sucrose between chloroplast and cytoplasm after nonaqueous extraction of leaves which had been illuminated in $^{14}CO_2$ showed that some of the sucrose was in the chloroplast fraction [5–7] but it was concluded from kinetic measurements [6] that the cytoplasm was probably the major site of sucrose synthesis. The chloroplast envelope was shown to be relatively impermeable to sucrose [8] and it seemed that if sucrose was synthesised in the chloroplast, it would not be transported out into the cytoplasm and so could not be exported from the leaf.

There is one report of isolated chloroplasts synthesising sucrose from CO_2 but this appeared to be a seasonal effect and could not be repeated at will [9]. One possible explanation for this result comes from [10] where, in normal chloroplast preparations, a small proportion of chloroplasts was found bounded by a layer of cytoplasm. When these bodies were separated from the rest of the preparation they synthesised sucrose and it seems likely that under some conditions the proportion of these structures in chloroplast preparations could by high enough to give misleading results.

We have developed a method for rapidly separating the chloroplast and cytoplasmic fractions of protoplasts with little cross contamination of the two fractions [11]. Using this technique we have determined the distribution of sucrose following photosynthesis by protoplasts in $^{14}CO_2$ and the results suggest that sucrose is neither synthesised in nor readily transported into chloroplasts. A preliminary report of this work has been published [12].

2. Materials and methods

Protoplasts were isolated from 7–9 days old wheat leaves and purified on a sorbitol–sucrose step gradient as in [13]. Photosynthesis was measured by addition of protoplasts to a medium containing 400 mM sorbitol, 1 mM $CaCl_2$, 25 mM Tricine (pH 7.6) and 5 mM $NaH^{14}CO_3$ (60 Ci/mol) to give 50 μg chl/ml. The mixture was illuminated in an oxygen electrode at 20°C as in [13] and incorporation of ^{14}C was determined as in [14]. Sucrose was separated by paper chromatography in ethyl acetate:pyridine:H_2O (8:2:1) and radioactivity was determined with a Berthold LB280 scanner. Label in sucrose–phosphate was determined by chromatography of samples treated with alkaline phosphatase. The identity of sucrose was always confirmed by chromatography of samples treated with invertase to ensure that quantitative conversion into glucose and fructose occurred. Chlorophyll (chl) was determined by the method in [15].

The procedure for rapid separation of the chloroplast and cytoplasmic fractions of protoplasts is

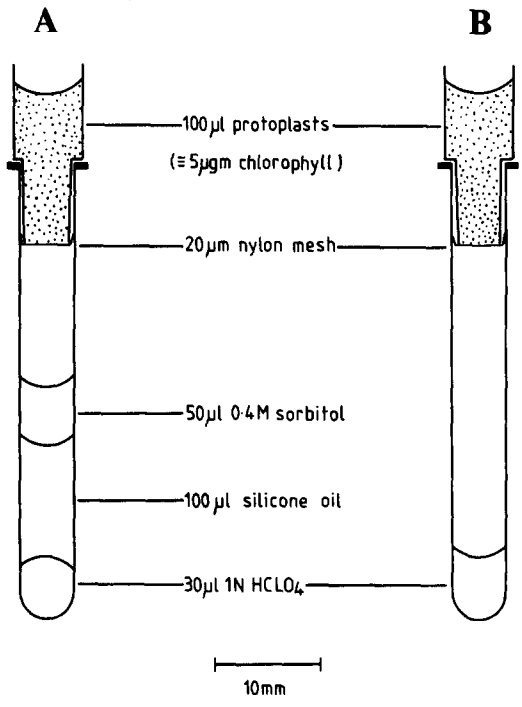

Fig.1. Diagram showing the method for separating chloroplast and cytoplasmic fractions of protoplasts. The protoplasts are held above a piece of 20 µm aperture nylon mesh in an adaptor inserted into a 0.4 ml. microcentrifuge tube. When the centrifuge is started the protoplasts rupture as they pass through the mesh but 85–90% of the chloroplasts released remain intact and sediment through the silicone oil layer leaving the remainder of the extract in the supernatant (A). Complete separation is achieved in < 6 s and contamination of the pellet fraction by mitochondria and peroxisomes is < 15% and by soluble cytoplasm < 5% [11]. A total protoplast extract is prepared simultaneously in a tube lacking the silicone oil layer (B).

described in fig.1 and further detailed [11]. Protoplasts were illuminated under the same conditions as for oxygen evolution measurements and illumination was continued during the 15 s centrifugation. Perchloric acid (final conc. 0.3 N) was added to the supernatant immediately after the centrifugation and the tubes were placed on ice. The supernatant fraction was removed and then the tubes were cut through the silicone oil layer and the pellet fraction was mixed and removed with a syringe. Both fractions were centrifuged to remove protein then neutralised with K_2CO_3.

3. Results

When suspensions of wheat protoplasts were illuminated, photosynthetic oxygen evolution and CO_2 fixation commenced after an induction period [16] and maximum rates were achieved after 6–8 min and maintained for ⩾ 15 min (fig.2). Illumination of the protoplasts in $^{14}CO_2$ and subsequent chromatography showed that 50–70% of the $^{14}CO_2$ fixed was incorporated into sucrose once a linear rate of photosynthesis was established and in this respect the protoplasts closely resembled whole leaves. Chloroplasts prepared from the protoplasts fixed CO_2 at comparable rates [13] but no incorporation of $^{14}CO_2$ into sucrose was detected in agreement with the results generally obtained with isolated spinach chloroplasts [9,10]. To establish the distribution of the labelled sucrose in protoplasts rapid separation of the chloroplast and cytoplasmic fractions was made using the method in fig.1. The results of 6 experiments where protoplasts were fractionated following photosynthesis in $^{14}CO_2$ for 10 min are given in table 1. The recovery of labelled sucrose in the two fractions was compared with a total protoplast extract prepared simultaneously to determine whether metabolism in the supernatant fraction could bias the

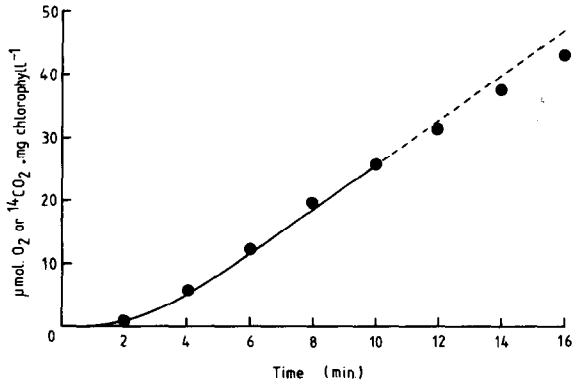

Fig.2. Time course of simultaneous oxygen evolution and $^{14}CO_2$ fixation by a suspension of wheat protoplasts. The circles indicate $^{14}CO_2$ fixed and the curve indicates oxygen evolved. Oxygen measurements were not continued after 10 min because the oxygen concentration was too high to ensure a linear electrode response. Measurements were made as in section 2 and the maximum rate of oxygen evolution was 210 µmol. mg chl^{-1}. h^{-1}.

Table 1
Distribution of sucrose in wheat protoplasts during steady state photosynthesis

Expt.	Rate of CO_2 fixation	% Sucrose in			Recovery of sucrose	% Sucrose in S
		P	S	P+S		
1	96	1.5	59	50	96	99.6
2	83	3.9	74	67	99	99.4
3	144	1.9	59	53	93	99.7
4	130	3.0	66	58	109	99.4
5	132	3.8	66	60	117	99.4
6	150	3.3	66	59	97	99.4
Mean	123	2.9	65	58	102	99.5
SD	27	1.0	5.6	6.0	9.2	0.13

The protoplasts were fractionated after 10 min photosynthesis in $^{14}CO_2$ and labelled sucrose was measured in the pellet (P) and supernatant (S) fractions. Rate of CO_2 fixation is μmol.mg chl^{-1}.h^{-1}. Sucrose in each fraction is given as a percentage of the total label in that fraction. Recovery is the amount of sucrose in (P+S) as a percentage of sucrose in a total extract prepared simultaneously (fig.1B)

results. The recovery was very close to 100% (table 1) indicating that metabolism of sucrose in the time between rupture of the protoplasts and addition of acid to the supernatant (~20 s) was not significant.

After 10 min photosynthesis, only ~10% of the $^{14}CO_2$ incorporated was recovered in the pellet (chloroplast) fraction and of this, only 1–4% was in sucrose. When the sum of sucrose in the two fractions was calculated, > 99% was recovered in the supernatant (cytoplasm) fraction. In addition, sucrose formed a greater percentage of the label in the supernatant fraction than it did in the sum of the two fractions and this enrichment also indicates that sucrose was synthesised solely in the cytoplasm. When intact chloroplasts sediment through silicone oil a small layer of fluid outside of the chloroplast is carried through (possibly in the space between the two envelope membranes) and this space is permeable to sucrose [8] which could account for the small amounts of sucrose found in the pellet fraction. To measure this contamination, protoplasts in a medium with unlabelled CO_2 but with [^{14}C]sorbitol were fractionated and 0.2–0.5% of the sorbitol in the supernatant was found to be carried through into the pellet fraction. This result agrees with the percentage of sucrose recovered in the pellet fraction (table 1) indicating that the sucrose in the pellet fraction was probably not inside the inner envelope membrane of the chloroplasts. Essentially the same results were obtained with protoplasts isolated from spinach leaves.

Although the pathway for sucrose synthesis is thought to involve the formation and hydrolysis of sucrose phosphate [1,4], < 2% of the $^{14}CO_2$ fixed by wheat protoplasts was in sucrose phosphate suggesting that an active sucrose phosphate phosphatase reaction kept the pool of this metabolite low. No sucrose phosphate could be detected in the pellet fraction. It is possible that sucrose, or sucrose phosphate, could be synthesised in the chloroplast and rapidly transported into the cytoplasm so that sucrose accumulates in the cytoplasm (although this would be contrary to the known impermeability of the chloroplast envelope to sucrose [8]). To check this possibility, protoplasts were fractionated after varying times of photosynthesis and the distribution of sucrose was measured. Figure 3 shows the results of one such experiment and the oxygen evolution and CO_2 fixation for the same experiment is given in fig.2. Sucrose synthesis only commenced after 2–3 min and the percentage of the total $^{14}CO_2$ fixed which was recovered in sucrose rose until a steady level was reached after 8–9 min indicating a linear rate of sucrose synthesis. If sucrose was synthesised in the chloroplast, the percentage of sucrose in the supernatant fraction would be expected to extrapolate

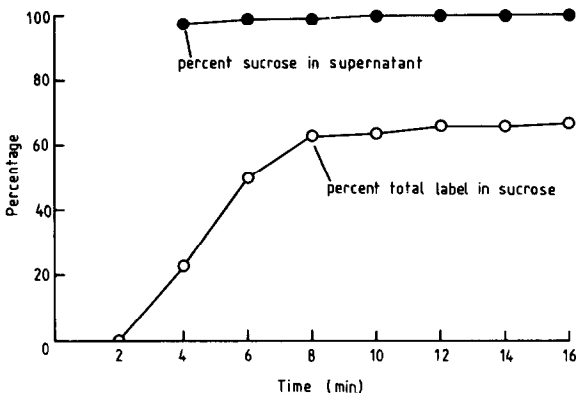

Fig.3. Time course of the incorporation of $^{14}CO_2$ into sucrose by wheat protoplasts. The data is from the same experiment as fig.2. The lower curve is the percentage of total $^{14}CO_2$ fixed which was incorporated into sucrose. In the upper curve, the sucrose recovered in the supernatant (cytoplasmic) fraction is plotted as a percentage of the total sucrose in pellet plus supernatant fractions.

to zero at time zero but, as shown in fig.3, even at the earliest time when sucrose was detected 97% was recovered in the supernatant fraction indicating the cytoplasm as the site of sucrose synthesis.

It is possible that the high proportion of sucrose in the supernatant fraction resulted from export of sucrose from the protoplasts into the medium. When the intact protoplasts were removed by low speed centrifugation, $< 10\%$ of the $^{14}CO_2$ fixed was in the medium after 20 min photosynthesis and this most probably resulted from breakage of protoplasts. This suggests that the products of photosynthesis were retained within the protoplasts.

4. Discussion

The present results strongly indicate that sucrose is not synthesised in the chloroplast and that sucrose synthesised in the cytoplasm does not readily penetrate beyond the inner chloroplast envelope membrane. Previous studies in which the enzymes of sucrose synthesis or labelled sucrose were found in chloroplast preparations were most probably the result of contamination by cytoplasmic material and this highlights the need for critical assessment of levels of contamination in studies of intracellular localisation. The results of protoplast fractionation cannot disprove the notion that sucrose synthesis might occur in some other organelle such as mitochondria or endoplasmic reticulum as these do not sediment through the silicone oil layer [11]. However, the soluble fraction of leaf homogenates contains $> 90\%$ of the activities of sucrose phosphate synthetase and UDP glucose pyrophosphorylase [4] and of sucrose phosphate phosphatase [17] which indicates that sucrose is synthesised in the soluble phase of the cytoplasm.

It seems most likely that sucrose is synthesised in the cytoplasm from triose phosphate which is exported from the chloroplast in exchange for an uptake of cytoplasmic orthophosphate [1,18]. The orthophosphate released during conversion of the triose phosphate to sucrose would maintain the cytoplasmic and ultimately the chloroplast orthophosphate pool to allow continued photosynthesis.

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