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PHOTOSYNTHESIS IN VITRO. I. ACHIEVEMENT OF HIGH RATES

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SUMMARY

Photosynthetic $^{14}\text{CO}_2$ fixation, at rates up to half the expected maximum in vivo rates for spinach leaves, has been achieved with a system reconstituted from washed, broken spinach chloroplasts and soluble components from previously isolated whole chloroplasts. This $^{14}\text{CO}_2$ fixation is strictly light dependent, and the resulting products are principally intermediate compounds of the reductive pentose phosphate cycle. This complete cycle is operating in the reconstituted system, since only the carboxylation product, 3-phosphoglycerate, is required as a primer.

INTRODUCTION

Light-induced carbon dioxide fixation by isolated spinach chloroplasts was demonstrated by Arnon et al. in 1954¹. Whatley et al. observed CO_2 fixation by osmotically ruptured chloroplasts in 1956², and Trebst et al.³ demonstrated that a supernatant fraction, derived by centrifugation of sonically ruptured chloroplasts, was capable of catalyzing the conversion of ^{14}C -labeled bicarbonate into acid soluble compounds when NADPH and ATP were supplied. Park and Pon⁴ showed that

when a fraction containing washed broken chloroplast fragments was added to a fraction containing soluble enzymes from chloroplasts, light-induced CO_2 uptake into the sugar phosphate intermediate compounds of the reductive pentose phosphate cycle of photosynthesis occurred.

As a result of improvements made in experimental procedures by various workers, rates of fixation of CO_2 by isolated spinach chloroplasts gradually improved, reaching 50% or more of the rates in intact spinach leaves by 1966⁵. However, rates of photosynthesis of CO_2 by reconstituted systems of chloroplast fragments and soluble enzymes from chloroplasts do not appear to have approached in vivo rates, and it has been suggested that reconstitution of an envelope-free suspension capable of operating the entire reductive pentose phosphate cycle might not be possible⁶. However, such a system would be useful in studies of regulatory mechanisms of photosynthesis, since an in vitro system could make possible investigations without the added complications caused by transport across the outer chloroplast membrane. We report here the achievement of in vitro photosynthesis with CO_2 at rates up to 120 $\mu\text{moles CO}_2$ fixed per mg chlorophyll per hour--about 1/2 of the rate expected with active spinach leaves in vivo.

METHODS

Two batches of spinach chloroplasts are isolated according to the method of Jensen and Bassham⁵. For each batch, 11 g of freshly harvested spinach leaves are washed, the midribs are removed, and the leaves are cut into small pieces. Leaf pieces and 33 ml Solution A (Table I), precooled to 0°C are placed in a chilled small vessel (capacity about

100 ml) on a blender and are blended for only 5 sec at high speed. The slurry is poured through 6 layers of cheesecloth, and the resulting juice is centrifuged at $2000 \times g$ for 50 sec.

Each chloroplast pellet is then suspended in 5 ml of Solution Y (Table I) which is precooled to 0°C . (Both Solution Y and Solution Z, required below, should be readjusted to pH 8 just prior to use, since the buffering capacity of these solutions is limited, and the pH can be affected by atmospheric CO_2). After 2 min, the chloroplasts are again centrifuged at $2000 \times g$ for 50 sec. This step is required to remove residual soluble enzymes.

To disrupt the chloroplasts, the pellets from the two batches are each suspended in about 1.25 ml of Solution Z and are combined, and allowed to stand at 0°C for 10 min. The suspension is then centrifuged at $27,000 \times g$ for 10 min. The straw-colored supernatant solution containing the soluble components from the chloroplasts is stored at 0°C until the reconstitution experiment (see below), which is carried out within an hour.

The pellet is resuspended in 5 ml Solution Z for washing, and after 10 min is centrifuged at $27,000 \times g$ for 10 min. The pellet is stored at 0°C until needed. Just before the experiment it is suspended in 2.4 ml Solution Z.

For time course experiments, 800 μl of the soluble protein solution, containing 4 to 8 mg of protein, is placed in a 15 ml round bottom flask. To this is added 50 μl of the resuspended green pellet, containing 50 to 100 μg chlorophyll. In a typical experiment, the flask contained 58.6 μg chlorophyll and 7.0 mg soluble protein, for a weight ratio of 119/1.

The whole chloroplasts in the same experiment contained soluble protein/chlorophyll = 8.2. Thus the reconstituted system had a 14-fold greater ratio of soluble protein to chlorophyll.

Further additions are made so that the final contents of the flask contain added 3-phosphoglycerate (1.0 mM), spinach ferredoxin (100 µg), NADP (0.5 mM), ADP (0.1 mM) in a final volume of 1.0 ml. The flasks are closed with a serum stopper and attached to the shaker (described previously), with immersion of the flask in a bath at 20°C, and illumination through the transparent bottom of the bath with a bank of fluorescent 40 watt lights which provides an incident intensity of 25,000 lux⁵.

The flasks are illuminated with swirling for 5 min, at which time 100 µl of a solution of bicarbonate (0.15 mM, 20 µC/µmole) is injected through the serum cap. Illumination and shaking are continued, with the shaking periodically interrupted to allow 50 µl samples to be withdrawn with a microsyringe and hypodermic needle through the serum cap. These samples are immediately injected into 450 µl of methanol at room temperature to stop the biochemical reactions. In some time course experiments one flask was darkened after 10 min photosynthesis by shrouding it with black cloth, tightly gathered at the neck of the flask, which was also masked with black tape. After another 10 min the shroud was removed and photosynthesis was allowed to resume (see Results).

An aliquot portion of each killed sample is applied to a piece of filter paper along with a drop of glacial acetic acid, and a stream of N₂ is used to dry the spot. The ¹⁴C content of the compounds is measured by counting between opposing thin window G-M tubes. From the known tube sensitivity and specific radioactivity of the labeled bicarbonate, and

the previously determined chlorophyll content, the photosynthetic assimilation is calculated⁵.

The radioactive products of photosynthesis were analyzed by two-dimensional paper chromatography in phenol-acetic acid-water and in butanol-propionic acid-water as described elsewhere⁷. Radioactive compounds on the paper were detected by X-ray radioautography. When necessary, mixtures of sugar phosphates are eluted from the paper, treated with a phosphatase enzyme, and rechromatographed to separate the free sugars. The radioactive areas of the paper were cut out and the ^{14}C content was measured with opposing thin window G-M tubes mounted in the automatic "spot counter" as described before⁷. Data from this apparatus is automatically recorded on paper punch tape, along with typed-in experimental data, and the data are processed by computer to give $\mu\text{g-atoms } ^{14}\text{C}$ (per mg chlorophyll) in each compound.

RESULTS

As is the case with photosynthesis in isolated whole spinach chloroplasts, photosynthesis rates are strongly dependent on the health and physiological state of the leaves used in the preparation. With leaves from growth chambers, initial rates of photosynthesis from 30 to 90 $\mu\text{moles of CO}_2$ fixed per mg chlorophyll per hour were achieved. With leaves from plants grown outdoors in April, the highest rate we have seen so far was 120 $\mu\text{moles CO}_2$ fixed per mg chlorophyll per hour. In most cases the rates are essentially linear during the first 20 min, then decrease slowly to about 2/3 the initial value by 40 min (Fig. 1). Thus the rates are maintained somewhat better than in isolated whole chloroplasts.

The choice of "primer"--3-phosphoglycerate--was made so that the complete carbon reduction cycle would have to operate before the carbon in the "primer" could be incorporated into the immediate carboxylation substrate, ribulose-1,5-diphosphate. Such reduction of phosphoglycerate to sugar phosphates and conversion to carboxylation substrate does occur during the 5-min preincubation period, so that upon addition of ^{14}C -labeled bicarbonate, fixation is immediately observed, and the fixation rate remains constant for 20 min or more.

The amount of 3-phosphoglycerate present at the start, 1 mM in 1 ml, is 1.0 μmole for approximately 0.1 mg chlorophyll per flask, or 30 μg -atoms of carbon per mg chlorophyll. Since this is equivalent to 20 min fixation (when the rate is 90 μmoles of CO_2 fixed per mg chlorophyll per hour), there is considerable dilution of the administered ^{14}C incorporated by the system into sugar phosphates.

The fixation of $^{14}\text{CO}_2$ into acid-stable products is completely light-dependent (Fig. 1). After a period of 10 min darkness, the rate when the light is again turned on is about the same as it was when the light was turned off, even though the rate in the flask with continuous light had by this time declined somewhat. Thus it appears that the cause of the decline in rate after 20 min of photosynthesis is in some way related to the light processes, and is not merely the result of thermal degradation of enzymes.

As is the case with isolated whole spinach chloroplasts, nearly all of the incorporated ^{14}C is found in intermediate compounds of the reductive pentose phosphate cycle (Table II). Not surprisingly, the largest accumulation of ^{14}C is in 3-phosphoglycerate which is present as a primer at the

start. Substantial amounts of ^{14}C are found in hexose and heptose monophosphates, while the levels of ^{14}C in dihydroxyacetone phosphate and fructose-1,6-diphosphate are lower than in whole chloroplasts. Thus, with the conditions chosen, it appears that the reaction mediated by fructosediphosphatase is somewhat less rate limiting, and the reduction of 3-phosphoglycerate to triose phosphate somewhat more rate limiting than is the case with whole spinach chloroplasts.

In early experiments we found that broken chloroplasts, after the first centrifugation, still retained considerable ability to fix $^{14}\text{CO}_2$, despite the loss of most of the soluble enzymes. Thus, a wash step was instituted. A small amount of fixation still is found with the broken washed chloroplasts without soluble protein added back (Table III). Inspection under the optical microscope show the preparation to be well disrupted, with no intact chloroplasts visible. Presumably this residual activity is due to broken thylakoids reclosing and trapping soluble enzymes.

DISCUSSION

The demonstration that rates of photosynthesis with CO_2 up to half the maximum rates expected in vivo can be achieved with a reconstituted system of soluble enzymes and washed broken spinach chloroplasts from previously isolated chloroplasts shows that the compartmentalization provided by the limiting membrane of the whole chloroplast is not absolutely required for the operation of the reductive pentose cycle of photosynthesis at high rates. Having the soluble enzymes and intermediary metabolites uniformly distributed through the whole space of the

solution does not impose severe limitations due to diffusion times. Moreover, if there is any required organization of soluble enzymes with lamellae, it would have to be an organization that is easily and spontaneously reconstituted. In the absence of any evidence for such organization at present, a simpler hypothesis is that such organization is not required, and that intermediate compounds and cofactors freely diffuse from one soluble enzyme to another. These alternatives will be amenable to testing with reconstituted systems employing various ratios and dilutions of soluble and membrane components.

This reconstituted system should prove useful in further investigations of the regulatory mechanisms proposed for photosynthetic carbon metabolism⁸. Already we have tested the effects of the spinach leaf regulatory factor which has such a large effect on rate of photosynthesis in whole chloroplasts and rate of movement of intermediary metabolites out of whole chloroplasts⁹. It was expected that this factor affects the outer chloroplast membrane and not the internal reactions of the chloroplast. If so, the rate and products of $^{14}\text{CO}_2$ fixation in the reconstituted system should not be affected by the factor. Preliminary experiments indicate that the factor (which has been separated from fructose-1,6-diphosphatase) is without effect on the reconstituted system.

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TABLE I

SOLUTIONS USED IN PREPARATION OF RECONSTITUTED SYSTEM

All concentrations are given as millimolar.

Component	Solution A	Solution Y	Solution Z
Sorbitol	330	330	0
KNO ₃	2	0	2
EDTA (K ₂ salt)	2	2	2
Na isoascorbate	2	2	0
MnCl ₂	1	1	1
MgCl ₂	1	1	30
K ₂ HPO ₄	0.5	0	0.5
NaCl	20	0	0
Na ₂ P ₂ O ₇	0	5	0
Glutathione	0	50	50
MES*	50	0	0
Dithiothreitol	0	0	1
Enough NaOH to bring pH to	6.1	8.0	8.0

*2-(N-morpholino)ethanesulfonic acid

TABLE II

ACCUMULATION OF ^{14}C IN METABOLITES IN RECONSTITUTED CHLOROPLAST SYSTEM

All amounts are in $\mu\text{g-atoms}$.

Compound	Time: 9 min	19 min	28 min
3-phosphoglycerate	4.00	9.01	13.02
glucose-6-phosphate plus sedoheptulose-7-phosphate	2.51	4.87	6.08
fructose-6-phosphate	0.86	1.43	1.45
dihydroxyacetone phosphate	0.47	0.55	0.54
fructose-1,6-diphosphate	0.18	0.19	0.17
sedoheptulose-1,7-diphosphate plus glucose-1,6-diphosphate	0.23	0.36	0.36
ribulose-1,5-diphosphate	0.06	0.08	0.09
adenosine diphosphoglucose	-	0.09	0.41
starch	0.01	0.02	0.06
Total (major labeled "spots" on chromatograms)*	8.32	16.60	22.18
Total acid-stable fixation	9.33	18.85	26.02

*Some of difference between this total and total fixed is distributed among minor spots, including pentose monophosphates.

TABLE III

^{14}C INCORPORATION BY WASHED BROKEN CHLOROPLASTS, BY SOLUBLE
COMPONENTS, AND BY RECONSTITUTED SYSTEM

	Relative rate
Reconstituted system	1.000
Washed broken chloroplasts	0.015
Soluble enzymes	0.016

FIGURE CAPTION

Fig. 1. Time course of $^{14}\text{CO}_2$ incorporation during light, and in light-dark-light experiment.

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