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PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS

IV. REGULATION BY FACTORS FROM LEAF CELLS

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SUMMARY

The rate of photosynthesis by isolated spinach chloroplasts is strongly affected by the presence of clarified juice from spinach leaves. With chloroplasts photosynthesizing under otherwise optimal conditions (which include the presence of 5 mM pyrophosphate), the presence of juice in an amount which corresponds to 1/4 of the calculated ratio *in vivo* of juice/chloroplasts causes a 20% stimulation in the rate, whereas 3/4 of the full calculated amount causes 90% inhibition.

The degree of inhibition or stimulation is a complex function of the ratio of added pyrophosphate to added juice. With a wide range of concentrations of added juice, additions of small amounts of pyrophosphate cause the rate of photosynthesis to decrease, and this inhibition increases with increasing pyrophosphate concentration, becoming and remaining severe until a threshold ratio of pyrophosphate to juice is reached. This threshold ratio is nearly constant over a 12-fold range of added juice. Beyond the threshold ratio, increasing pyrophosphate (at a given level of juice) reverses the inhibition, and with roughly 3 times the threshold ratio, the control rate is surpassed and stimulation is observed.

Gel filtration of the juice produces two well-separated inhibitory fractions. One inhibitory fraction contains a compound of high molecular weight complexed with a small molecule, while the second inhibitory fraction contains the unassociated small molecule. The complex is inhibitory only in the presence of pyrophosphate, whereas the small unassociated molecule has the same activity whether pyrophosphate is present or not. The complex is stabilized in the presence of pyrophosphate. Without pyrophosphate present, the complex dissociates almost completely into its constituents. Dialysis of the dissociated complex gives an inactive large molecule in the retentate. The activity of the small molecule is greatly amplified when it is added back to the inert large molecule.

The inhibitory fractions are found, at least in part, in previously isolated chloroplasts.

These findings and other results described in the text strongly suggest a role for these factors in the regulation *in vivo* of photosynthesis.

Abbreviations: MES, 2-(*N*-morpholino)-ethanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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INTRODUCTION

Previous studies of photosynthesis by isolated spinach chloroplasts in the presence of $^{14}CO_2$ and $^{32}P_i$ have established several characteristics. High rates of photosynthesis are possible, and are stimulated by added inorganic pyrophosphate[1]. Some intermediate compounds of the photosynthetic carbon reduction cycle readily diffuse out of the chloroplasts, whereas other intermediate compounds are well retained[2]. With isolated chloroplasts photosynthesizing at high rates (greater than 100 μmoles of CO_2 fixed per mg chlorophyll·h) no stimulation of the photosynthetic rate was found upon the addition of any of several intermediates of the photosynthetic carbon reduction cycle[3]. No stimulation was seen upon the addition of ATP, ADP, AMP or $NADP^+$. Little conversion of intermediates of the photosynthetic carbon reduction cycle to secondary metabolic compounds such as amino acids and free sugars was seen[1]. It appeared that substances required for such conversion were missing from the isolated chloroplasts.

Preliminary studies showed that addition of clarified spinach juice caused inhibition of the rates of photosynthesis by isolated chloroplasts. Several fractions have now been obtained which have large effects on the rate of CO_2 photosynthesis by isolated chloroplasts. Certain characteristic properties of the inhibitory fractions described below suggest that they contain factors which play a role in the regulation of photosynthesis.

METHODS AND MATERIALS

Spinach leaves are harvested from a nearby farm and brought on ice to the laboratory soon after being picked. The leaves are stored at 0° for as long as 7 days.

Buffers and biochemical compounds are purchased from Calbiochem., Los Angeles, Calif. Buffers include 2-(*N*-morpholino)-ethanesulfonic acid (MES) and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES).

Each of the three solutions used in the isolation and assay of chloroplasts[1] contains the following: 0.33 M sorbitol, 0.002 M $NaNO_3$, 0.002 M EDTA (dipotassium salt), 0.002 M sodium isoascorbate (added on day of use), 0.001 M $MnCl_2$, 0.001 M $MgCl_2$ and 0.0005 M K_2HPO_4.

In addition, Soln. A contains 0.02 M NaCl, 0.05 M MES, adjusted with NaOH to pH 6.1; Soln. B contains 0.02 M NaCl, 0.05 M HEPES, adjusted with NaOH to pH 6.7; Soln. C contains 0.005 M $Na_4P_2O_7 \cdot 10\ H_2O$ (added on day of use), 0.05 M HEPES, adjusted with NaOH to pH 7.6. All solutions are stored at 4°.

Spinach leaf juice

Spinach leaf juice is prepared with a juicer (Model 6001, Acme Juicer Mfg. Co., Sierra Madre, Calif.). This apparatus contains a flat circular grater which rotates at high speed and against which the leaves are pressed. Surrounding the rotating grater is a rotating screen against which the shredded leaves and juice are thrown by centrifugal action. This apparatus is precooled to 4°, and the juice emerging from the apparatus is collected in a chilled tube. Usually, 0.2 ml of Soln. C *minus* PP_i is mixed with each gram of leaves as they are shredded by the juicer. About 0.7 ml juice is

obtained per gram of leaves. This is adjusted to pH 7.6 with NaOH, and the juice is then clarified by centrifugation at 200000 × *g* for 1 h.

Chloroplast preparation

In a cold room, at 4°, 12 g of leaves are washed and cut after removing the midribs. The leaf strips and 33 ml chilled Soln. A are placed in a "semimicro" Monel container on a Waring blender, Model 1120, and blended for 5 sec at high speed. The slurry is poured and pressed through 6 layers of cheese-cloth (42 threads/inch), and the resulting juice is centrifuged for 50 sec at 2000 × *g*. The chloroplasts in the resulting pellet are resuspended in Soln. B at 0° to give a suspension which contains 2–3 mg chlorophyll per ml. This suspension is used for assay within 2 h.

Extract from sonicated chloroplasts

Chloroplast extract is prepared from chloroplasts which are isolated from spinach leaves as described above, with the following modifications: The leaves are ground in Soln. B instead of Soln. A for 7 sec. Centrifuging is carried out for 2.5 min instead of 50 sec. 12 pellets of chloroplasts, each obtained from 12-g batches of spinach leaves, are resuspended in 9 ml chilled Soln. C *minus* PP_i.

The suspension is placed in a beaker, cooled in crushed ice, and sonicated for 4 periods of 15 sec, using a precooled probe (Biosonik, Model BP 1, Bronwill Scientific, Rochester, N.Y.). 10-min intervals between the sonication periods are allowed to avoid overheating. The resulting broken chloroplast suspension is checked for complete destruction of chloroplasts by phase microscopy, adjusted with NaOH to pH 7.6 and clarified by centrifugation at 200000 × *g* for 1 h.

Assay of photosynthesis rate

Photosynthesis is carried out in 15-ml round-bottom flasks stoppered with serum caps. 25 µl of the chloroplast suspension are injected into each flask containing 450 µl of the medium to be assayed.

The basic medium is either complete Soln. C or Soln. C deficient in either Mg^{2+} or PP_i, or both. In those experiments where the medium contains juice or portions of fractions from gel filtration or dialysis, mixing is done in such a way as to keep the concentrations of all constituents present approximately constant. The amounts of juice or fractions added in various experiments are shown in the figures. In some experiments the medium is the effluent of the gel filtration.

The medium is kept at 4° until the assay is started. Assays are conducted with flasks mounted on a shaking rack and illuminated from below in a constant temperature bath, as described before[1].

The chloroplasts in the swirled flasks are preilluminated for 5 min and are allowed to synthesize with ^{14}C-labeled bicarbonate for 10 min. The reaction is terminated by the addition of 4.5 ml of methanol to each flask.

For determination of total ^{14}C fixed, an aliquot sample of this chloroplast–methanol mixture is spotted on a piece of filter paper, acidified with acetic acid, dried, and its radioactivity measured with Geiger–Müller tubes. Alternatively, radioactivity is measured by scintillation counting. From the known counter sensitivities, and the specific activity of the ^{14}C used, the amount of CO_2 fixed in the 10-min period is calculated.

Another aliquot sample of the original spinach chloroplast suspension is extracted with 80% acetone for the spectrophotometric determination of chlorophyll content[4]. This figure *plus* the calculated amount of CO_2 fixed permits the calculation of the rate of photosynthesis (μmoles CO_2 fixed per mg chlorophyll·h).

RESULTS

Fig. 1 shows the effect on photosynthesis rate by isolated spinach chloroplasts of adding various amounts of spinach juice, in the presence and absence of Mg^{2+} (1 mM) and of pyrophosphate ion (5 mM). The control rate of 1.0 on the vertical scale (128 μmoles CO_2 fixed per mg chlorophyll·h) is obtained with Mg^{2+} and PP_i present (the normal complete medium) and no juice added. In the presence of pyrophosphate, 5–15 μl of juice cause a significant enhancement of the rate, either in the presence or absence of Mg^{2+}. With increasing amounts of added juice there is a dramatic drop in the photosynthesis rate. With the addition of 30 μl of juice, the rate is reduced to about 5% of the control rate, where it remains with subsequent additions of juice up to 80 μl.

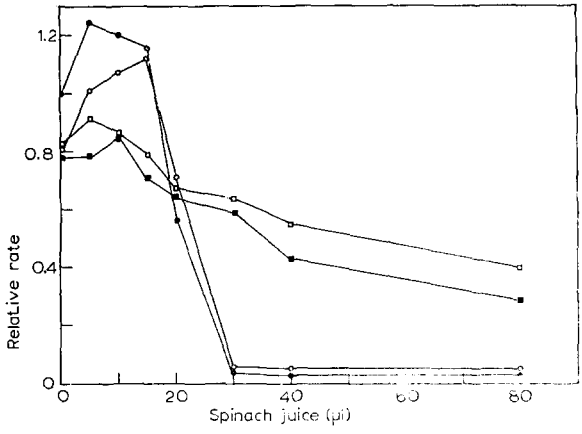

Fig. 1. Dependence of photosynthetic CO_2 fixation rate in isolated chloroplasts on the amount of added spinach juice in presence and absence of Mg^{2+} and PP_i. Each flask contained chloroplasts with 73 μg chlorophyll. Control rate (1.0 on vertical scale) was 128 μmoles CO_2 fixed per mg chlorophyll·h. Control was conducted with complete Soln. C as medium. □—□, Soln. C *minus* PP_i and Mg^{2+}; ■—■, Soln. C *minus* PP_i; ○—○, Soln. C *minus* Mg^{2+}; ●—●, complete Soln. C.

A striking difference is seen upon the omission of PP_i, either in the presence or absence of Mg^{2+}. In this case there is little enhancement, and with larger amounts of juice the inhibition is much less pronounced. Thus we see the potentiating effect of PP_i on the enhancement and particularly on the inhibition by added juice.

The more active spinach leaves have been found to contain approx. 0.9 mg chlorophyll per g leaf[1]. When juice is prepared as described in METHODS AND MATERIALS, about 0.5 ml undiluted juice is obtained per gram of leaf, which gives a value of 0.55 ml juice per mg chlorophyll. Since there is 73 μg chlorophyll in each flask in the experiment shown in Fig. 1, the amount of juice corresponding to the ratio *in vivo* is 40 μl.

Fig. 2a gives the rate of photosynthesis by isolated chloroplasts relative to the

control as a function of various amounts of added juice in the presence of various concentrations of PP_i from 0 to 20 mM. In this experiment, 0.2 ml of Soln. C *minus* PP_i was added per gram of leaves as the juice was being prepared, so that 0.68 ml diluted juice was obtained from each gram of leaves. In this case, therefore, there was 0.75 ml juice per mg chlorophyll. Since there was 56 μg chlorophyll in each flask, the amount of juice per flask, which would again be equivalent to the approximate amount *in vivo*, is 42 μl.

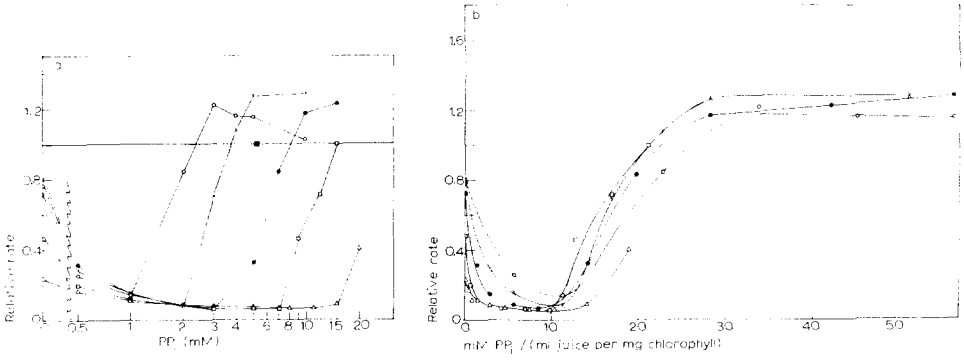

Fig. 2. (a) Dependence of photosynthetic CO_2 fixation rate in isolated chloroplasts on the concentrations of added spinach juice and PP_i. 56 μg chlorophyll per flask. Control (complete Soln. C): 84 μmoles CO_2 fixed per mg chlorophyll·h. (b) Relative rate of photosynthetic CO_2 fixation as a function of the ratio of concentration of added pyrophosphate to the amount of added juice per mg chlorophyll. Plotted points are derived from data shown in Fig. 2a. 40 μl juice per flask corresponds to 0.71 μl spinach juice per mg chlorophyll, *i.e.* the estimated concentration *in vivo*. ■, no juice (control); μl juice per flask: ○—○, 5; ×—×, 10; ●—●, 20; □—□, 40; △—△, 60.

The results in this experiment show clearly the importance of the ratio of PP_i concentration to amount of added juice in producing stimulation or inhibition of the photosynthetic rate. We have used the amount of juice in each flask corrected to ml juice per mg chlorophyll to calculate the ratio of mM PP_i to ml juice per mg chlorophyll from the various points on the curves shown in Fig. 2a. The relative rate of photosynthesis is plotted against this ratio in Fig. 2b. With each level of added juice, increasing PP_i concentration causes increasing and then maximum inhibition until a certain threshold ratio is reached. The threshold ratios of mM PP_i concentration: ml juice added per mg chlorophyll range from about 10 to 12 for 5 μl to 40 μl added juice, and 14 for 60 μl of juice.

Once the threshold ratio of PP_i/juice is passed the rate of photosynthesis increases rapidly with increasing PP_i concentration. The maximum rates (>120% of control) are reached when the ratio of PP_i/added juice is very roughly 3 times the threshold ratio, with 5–20 μl of added juice.

In another experiment, the results of which are not shown, it was found that when juice is prepared from only the stems of spinach leaves, some enhancement (up to 20%) but no inhibition is seen in the complete medium, which contains PP_i and Mg^{2+}. Thus, it seems clear that the inhibitory factor is a constituent of the leaf cells only.

Inhibitory factors can be partially resolved if the spinach juice is placed on a column containing Sephadex G-25, previously equilibrated with complete Soln. C,

and eluted with this solution into fractions, portions of which are subsequently used as media for the assay of the photosynthetic rate (Fig. 3). The first fraction which exhibits strong inhibitory activity (labeled Fraction A) comes off the column just after an effluent volume corresponding to the void volume of the Sephadex bed. Subsequent fractions are slightly stimulatory, or neutral. Then follows a second inhibitory fraction (labeled Fraction B). The volume of elution of these two fractions is such as to suggest that Fraction A contains a compound of high molecular weight which is inhibitory, whereas Fraction B contains an inhibitory substance of smaller molecular weight.

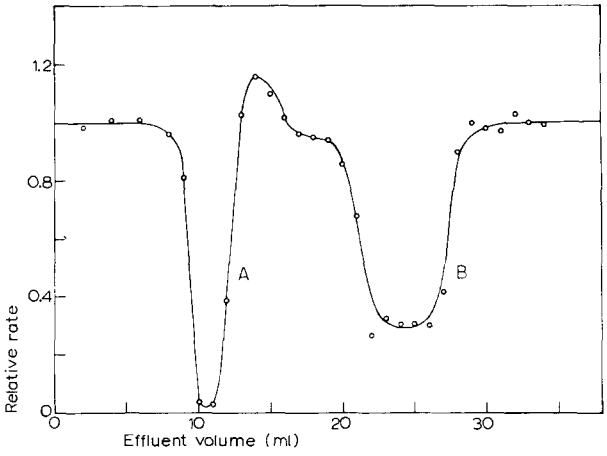

Fig. 3. Photosynthetic CO_2 fixation rates of isolated chloroplasts suspended in portions of effluent fractions of spinach juice separated by gel filtration on Sephadex G-25. 62 μg chlorophyll per flask. Control (complete Soln. C): 129 μmoles CO_2 fixed per mg chlorophyll·h.

When a similar experiment was performed, except that PP_i was omitted from elution and assay, inhibition was seen only with the fractions corresponding to Fraction B. After addition of PP_i to all fractions, inhibition nearly as great as that seen in Fig. 3 was now observed with those fractions corresponding to Fraction A. Thus the inhibitory activity of the presumed large molecule requires the presence of PP_i, whereas inhibition by Fraction B is not dependent on the presence of PP_i.

When Fraction A, obtained as described above (Fig. 3) and left at 4° for 18 h, was recycled through another column of Sephadex G-25, it was partially recovered as Fraction A, while another part of the inhibitory activity showed up as Fraction B. Thus the inhibitor of presumed smaller molecular weight can be derived from the inhibitory substance of higher molecular weight in Fraction A.

Dialysis of Fraction A against Soln. C *minus* PP_i gave a retentate which on subsequent analysis was found to be no longer inhibitory, even in the presence of PP_i. In contrast, dialysis of Fraction A against complete Soln. C (which contains PP_i) gave a retentate that was still highly inhibitory when assayed with complete Soln. C. It may be concluded that the presence of PP_i tends to stabilize a complex made up of an inert large molecule and a small molecule. Once this complex dissociates, the small molecule can be removed by dialysis.

The effects of additions to the assay medium of original juice and its dialysis fractions, *i.e.* retentate and diffusate, and recombined retentate and diffusate, all

assayed in the presence and absence of Mg^{2+} and PP_i, are shown in Table I (Expt. 1). The effect of PP_i on the inhibitory activity of the various components is seen. Also, the data show that the inhibitory power of the small molecule of the diffusate is greatly increased by the presence of the large inert molecule from the retentate. The reconstitution of inhibitory activity upon recombination of retentate *plus* diffusate suggests a reformation of inhibitory complex.

TABLE I

RELATIVE RATES OF CHLOROPLAST PHOTOSYNTHESIS IN PRESENCE OF SPINACH JUICE AND VARIOUS JUICE FRACTIONS

Assays were made with complete Soln. C (+ PP_i) or with Soln. C *minus* PP_i (- PP_i). Retentates were obtained by dialysis of spinach juice (Expts. 1 and 2) or chloroplast extract (Expt. 3) against a 100-fold volume of Soln. C *minus* PP_i. The diffusate (Expt. 1) was prepared by dialysis of spinach juice against a 9-fold volume of outer solution. Fraction B (Expts. 2 and 3) was obtained by gel filtration of spinach juice (see text and Fig. 3). Controls (1.0 relative rate, with complete Soln. C): 65 (Expt. 1); 68 (Expt. 2); 89 μmoles CO_2 fixed per mg chlorophyll·h (Expt. 3).

Expt. No.	*Addition to assay medium*	*PP_i*	*Relative rate*
1	a. Original juice, 40 μl	—	0.68
		+	0.06
	b. Retentate, 40 μl	—	1.38
		+	1.26
	c. Diffusate, equivalent to 40 μl of original juice	—	0.47
		+	0.43
	d. Recombined retentate (b) and diffusate (c)	—	0.80
		+	0.08
2	50 μl Fraction B	+	0.93
	100 μl Fraction B	+	0.54
	30 μl retentate	+	1.01
	100 μl retentate	+	0.76
	50 μl Fraction B, 30 μl retentate	+	0.74
	50 μl Fraction B, 100 μl retentate	+	0.12
	100 μl Fraction B, 100 μl retentate	+	0.07
3	100 μl Fraction B	+	1.02
	100 μl retentate of chloroplast extract	+	1.12
	100 μl Fraction B *plus* 100 μl of chloroplast extract	+	0.16

The effects of adding Fraction B and inactive retentate to the assay medium, separately and combined, are shown in Table I (Expt. 2). Although the small molecule in Fraction B inhibits by itself, this inhibition is amplified upon the addition of retentate. Thus Fraction B and diffusate exhibit similar potentiating effect, due to the presence of the small molecule in each.

An extract of sonicated, previously isolated chloroplasts was prepared (see METHODS AND MATERIALS). The strong inhibition upon combination of retentate from this extract with Fraction B from spinach juice (Expt. 3, Table I) suggests that the high molecular weight component of the inhibition complex is of the same nature in spinach juice and chloroplasts.

DISCUSSION

The dependence of the rate of photosynthesis in isolated chloroplasts on the amount of added clarified spinach juice in our usual assay medium is remarkable.

A 20% or more stimulation with 5–10 μl of juice is changed to a 95% inhibition with 30 μl of juice. The latter amount is probably less than the amount of juice in the leaves corresponding to the amount of chlorophyll in the flask to which the juice was added. A doubling in concentration of some constituents (which accompanies the doubling of added juice from 15 to 30 μl) is responsible for a 20-fold change in rate of photosynthesis. This large rate change with a small increase in the amount of added juice suggests a regulatory function of the juice. Such control might occur through the regulation of some enzymic activity or delicately poised process such as transport through a membrane.

This hypothesis gains support from the strong potentiation of the inhibitory effect by small amounts of pyrophosphate and by the strict dependence of the effect on the ratio of pyrophosphate to added juice.

Further indication of a regulatory function is found in the nature of the most effective inhibitory component, a dissociable complex which is both stabilized and activated by pyrophosphate. It appears to be a molecule of high molecular weight which by itself is inert and an activating compound of smaller molecular weight.

This smaller molecule, found in Fraction B and in the diffusate, seems to have some inhibitory activity by itself, but its inhibitory activity is greatly enhanced upon formation of the complex with the high molecular weight compound. It may be that this compound of smaller molecular weight only exhibits inhibitory activity by virtue of its ability to complex with the large molecule which could be present already in the isolated chloroplasts. The activation of the inhibitory complex on addition of Fraction B only to isolated chloroplasts presumably would require endogenous PP_i in the chloroplasts.

The isolated chloroplasts may have lost much of the small inhibitory molecule but retained some of the high molecular weight compound necessary for the potentiation of the activity of the small molecule. In support of this hypothesis, addition of Fraction B obtained from spinach juice, together with the retentate of the extract from sonicated, previously isolated chloroplasts, causes extensive inhibition.

The strict dependence of inhibitory activity on the ratio of pyrophosphate relative to the level of inhibitory substance suggests an important role for pyrophosphate in the operation of the regulatory mechanism *in vivo*. While it is often assumed that pyrophosphate, formed by biosynthetic reactions, is immediately hydrolyzed, this seems not always to be the case in photosynthesis. Previous studies with algae photosynthesizing under steady-state conditions[5] show that there is a measurable steady-state level of pyrophosphate which can fluctuate between light and dark.

Pyrophosphate is produced during biosynthetic reactions leading to carbohydrate formation and in the formation of other macromolecules. Since it is hydrolyzed at a rate which is small enough to permit steady-state levels to exist, it may serve as a kind of measure of biosynthetic or photosynthetic rate or possibly as a measure of the synthesis of one class of compounds (*e.g.* carbohydrates) at some particular subcellular site. Operating in conjunction with the as yet unidentified inhibitory factors discussed above, pyrophosphate level could in turn regulate photosynthesis. It is noteworthy that magnesium pyrophosphate complex inhibits the activity of fructose diphosphatase[6] (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11). This enzyme is known to be one of the light–dark regulated enzymes of photosynthesis[5,7,8].

The stimulation of photosynthesis in isolated chloroplasts[1] by 5 mM PP_i in the absence of any added juice is probably due to the removal of inhibition caused by inhibitor complex already present in the isolated chloroplasts. From the results in Fig. 2, it is seen that 5 mM PP_i would bring the PP_i/juice ratio well beyond the threshold ratio for any small amounts of inhibitor that might have remained with the isolated chloroplasts.

Although inhibitory regulation, by itself, may not appear to be a "useful" function for metabolism, it must be remembered that the isolated chloroplasts do not represent the complete metabolic system of the cell. In particular, isolated chloroplasts make only a limited quantity of secondary products. Also, photosynthetic carbon reduction cycle intermediates which diffuse easily from the chloroplasts[2] are greatly diluted in concentration in the suspending medium, whereas in the intact cell they would be contained in a volume only several times the chloroplast volume. Thus, a change in the activity of a regulated enzyme of the carbon reduction cycle might lead to inhibition in isolated chloroplasts, whereas the same change *in vivo* could lead to a redistribution of carbon to secondary biosynthetic pathways.

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