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PHOTOSYNTHESIS OF ISOLATED CHLOROPLASTS

V. EFFECT ON FIXATION RATE AND METABOLITE TRANSPORT FROM THE CHLOROPLASTS CAUSED BY ADDED FRUCTOSE-1,6-DIPHOSPHATASE

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SUMMARY

Addition of fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11) *plus* Mg^{2+} in increasing concentrations causes stimulation and then inhibition of photosynthesis in isolated spinach chloroplasts. The effects are similar to those previously found with leaf extract additions, including modifications of the effects caused by added PP_i. Under the conditions of pH, Mg^{2+} concentration, and PP_i concentration in which these effects are seen, the added enzyme has only negligible activity as fructose-1,6-diphosphatase. Thus the enzyme appears to be exerting its effect through some function other than as an fructose-1,6-diphosphatase. It is tentatively suggested that this other function is in the selective diffusion of metabolites out through the chloroplast outer membrane. This hypothesis is supported by the following observations:

1. Addition of fructose-1,6-diphosphatase *plus* Mg^{2+} to chloroplasts photosynthesizing with $^{14}CO_2$ causes an increase in the total labeled Fru-1,6-P_2 and dihydroxyacetone-*P* (which appear mostly in the suspending medium), and a decrease in the total labeled Fru-6-*P* and of all compounds following Fru-1,6-P_2 in the carbon reduction cycle, including the carboxylation substrate, Ribul-1,5-P_2. Furthermore, the amounts of all metabolites retained in the chloroplasts decrease.

2. Addition of Mg^{2+} alone, in concentration slightly higher than PP_i concentration, causes some loss of all compounds from the chloroplasts to the medium.

3. Addition of 5 mM PP_i decreases the loss of hexose and heptose monophosphates and of Ribul-1,5-P_2, but does not decrease the loss of Fru-1,6-P_2 and of dihydroxyacetone-*P* from the chloroplasts to the medium.

INTRODUCTION

The rate of photosynthetic fixation of CO_2 in isolated spinach chloroplasts is strongly affected by additions of small amounts of clarified spinach juice[1]. The degree

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of inhibition or stimulation is a function of the ratio of amount of added spinach juice to the concentration of added PP_i. Fractionation of the juice by gel filtration gave two components which act synergistically to produce the stimulation or inhibition. The component of high molecular weight was identified as fructose-1,6-diphosphatase (D-fructose-1,6-diphosphate 1-phosphohydrolase, EC 3.1.3.11)[2]. The component of low molecular weight, called "Factor B" in the previous reports[1,2], has now been found to be an impure mixture of inorganic salts, of which the active constituent is Mg^{2+}. The synergistic stimulation and inhibition, and their dependence on PP_i, has been duplicated by additions to photosynthesizing chloroplasts of selected ratios of fructose-1,6-diphosphatase and Mg^{2+}.

Addition of Fru-1,6-P_2 to relatively inactive isolated spinach chloroplasts stimulates their fixation rate[3], while its addition to more active chloroplasts maintains a higher rate after 15 min of photosynthesis, when the rate begins to decline[4]. Other tested metabolites were generally inhibitory or without effect[4]. Thus the level of Fru-1,6-P_2 in chloroplasts might play some role in the regulation of the carboxylation of Ribul-1,5-P_2 to give 3-*P*-glycerate.

The carboxylation reaction and the conversion of Fru-1,6-P_2 to Fru-6-*P*, as well as the conversion of Sed-1,7-P_2, appear to be metabolically regulated steps in the photosynthetic carbon reduction cycle[5-9]. Both the carboxylase and the diphosphatases, when isolated and purified, exhibit a dependence of activity on pH and Mg^{2+} concentration[10,11] which could account at least in part for their regulation *in vivo*[12]. Among the labeled metabolites formed in isolated spinach chloroplasts photosynthesizing with $H^{14}CO_3^-$, there are pronounced differences in the rates of movement out of the chloroplasts into the suspending medium[13]. Those compounds which just precede the reactions mediated by the diphosphatases (Fru-1,6-P_2, dihydroxyacetone-*P* and glyceraldehyde-*P*) are found to a much greater extent outside the chloroplasts after a few minutes photosynthesis, whereas the hexose and heptose monophosphates, including Fru-6-*P*, and Ribul-1,5-P_2, are very well retained. If Fru-1,6-P_2 moves out of the chloroplasts by means of facilitated transport, a part of the apparent regulation of fructose-1,6-diphosphatase *in vivo* could be in fact a regulation of this transport, provided fructose-1,6-diphosphatase in the cytoplasm is less active than that inside the chloroplasts.

We have now investigated the effects of fructose-1,6-diphosphatase, Mg^{2+} and PP_i, added together and separately, on the levels of labeled metabolites formed in photosynthesizing chloroplasts, and the effects of these additions on the movement of metabolites out of the chloroplasts. Most of the effects reported were under conditions of pH, Mg^{2+} concentration and PP_i level under which the added fructose-1,6-diphosphatase could have little or no activity for the conversion of Fru-1,6-P_2 to Fru-6-*P*. The effects in the medium of the added fructose-1,6-diphosphatase seem therefore to be indicative of another activity of this protein.

EXPERIMENTAL

Chloroplasts were prepared from fresh spinach as described previously[14]. Kinetic studies of the formation of labeled compounds and their movement from the chloroplasts to the medium during photosynthesis by the isolated chloroplasts with $H^{14}CO_3^-$ were as described earlier[13].

In some experiments, an attempt was made to remove enzymes external to the chloroplasts by "washing". The chloroplast pellet from the initial centrifugation[14] was resuspended in 10 ml Solution B or Solution C and again centrifuged at 2000 × *g* for about 50 sec, after which the chloroplasts were stored for less than 10 min at 0° until the beginning of the illumination at 20°.

In those experiments in which the distribution of metabolites between chloroplasts and medium was to be determined, a 50-μl sample of the suspension of photosynthesizing chloroplasts was removed at the indicated times, and this sample was subjected to 10 sec of centrifugation with a Spinco microfuge. The pellet and the supernatant solution were separated and each was killed in 80 % methanol. The elapsed time from sampling to killing was about 25 sec. Some samples were killed immediately, without separation of chloroplast from medium, to permit subsequent evaluation of metabolite conversion during centrifugation.

The supernatant solution could not be completely separated from the pellet, and a contamination of the pellet by 10 % of the supernatant solution is estimated. In those cases where a very large portion of a given compound is found in the supernatant solution, a correction has been made in the results. This correction, equal to 0.10 of the ^{14}C found in the compound in the supernatant solution, is subtracted from the value found for the pellet and added to the value found for the supernatant solution.

For determination of the rates of photosynthetic incorporation of $^{14}CO_2$, aliquot samples of the killed chloroplasts–methanol–water mixtures were dried on filter paper, free CO_2 was driven off by addition of acetic acid, and the ^{14}C content was measured with a Geiger–Müller tube and scaler. Chlorophyll content was determined in a sample of the chloroplast suspension, and rate of photosynthesis was calculated from known specific radioactivity of $H^{14}CO_3^-$ used, ^{14}C content and chlorophyll content[14]. Products of photosynthesis were analyzed by two-dimensional paper chromatography and autoradiography, as previously described[5,6].

A solution of purified fructose-1,6-diphosphatase was prepared from isolated spinach chloroplasts as reported earlier[2]. The eluate from the gel filtration through Sephadex G-200 was dialyzed against "Solution C"[14] *minus* PP_i and ascorbate, and the dialyzed enzyme solution was diluted 25 times in Solution C *minus* ascorbate and PP_i. Amounts of this diluted solution, containing up to 30 μg of protein, were added to the chloroplasts in the illumination flasks, and the suspensions were made up to 0.5 ml, as indicated in RESULTS. Other additions of Mg^{2+}, PP_i, *etc.* are given under RESULTS.

RESULTS

The rate of photosynthesis by isolated spinach chloroplasts is stimulated 25 % by the addition of small amounts of Mg^{2+} and purified fructose-1,6-diphosphatase and is strongly inhibited by the addition of greater amounts of Mg^{2+} and fructose-1,6-diphosphatase (Fig. 1). The shape of this stimulation–inhibition curve and its dependence on the presence of 5 mM PP_i are exactly analogous to the effects earlier observed upon the addition of various amounts of spinach juice to the isolated photosynthesizing chloroplasts[1].

The inhibition of photosynthetic rate by Mg^{2+} and fructose-1,6-diphosphatase is reversed by higher levels of PP_i (Fig. 2). As was the case with spinach juice[2], or with fructose-1,6-diphosphatase *plus* Factor B, the concentration of added PP_i required to

reverse inhibition is proportional to the amount of inhibiting substances added. Thus, it is clear that the effects formerly observed upon adding small amounts of spinach juice to the isolated chloroplasts, including dependence on PP_i concentration, can be reproduced by the addition of purified fructose-1,6-diphosphatase and Mg^{2+}.

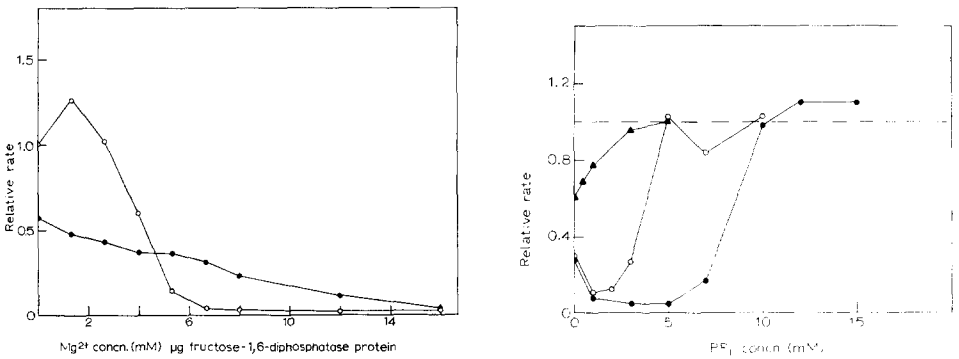

Fig. 1. Stimulation and inhibition of $^{14}CO_2$ fixation in isolated spinach chloroplasts by added fructose-1,6-diphosphatase and Mg^{2+}. ○, 5 mM PP_i; ●, no PP_i. Relative rate of 1.0 obtained with Solution C (5 mM PP_i and 1.0 mM Mg^{2+}) was 74.4 μmoles ^{14}C per mg chlorophyll per h. Mg^{2+} and fructose-1,6-diphosphatase were added to the reaction flasks (total volumes 0.5 ml) at the start of the preillumination period to give the concentrations indicated. After 5 min preillumination, $H^{14}CO_3^-$ was added and the chloroplasts were allowed to photosynthesize for 5 min.

Fig. 2. Dependence of inhibition of CO_2 fixation on concentration of added PP_i. ▲, 1 mM Mg^{2+}, no fructose-1,6-diphosphatase; ○, 3 mM Mg^{2+}, 4.2 μg fructose-1,6-diphosphatase; ●, 6 mM Mg^{2+}, 10.5 μg fructose-1,6-diphosphatase. Control rate of 1.0, with Solution C (1 mM Mg^{2+} and 5 mM PP_i) was 120 μmoles $^{14}CO_2$ fixed per mg chlorophyll per h. Other conditions as in Fig. 1.

TABLE I

EFFECTS OF Mg^{2+} AND OF FRUCTOSE-1,6-DIPHOSPHATASE ON RATES OF $^{14}CO_2$ UPTAKE BY ISOLATED SPINACH CHLOROPLASTS

PP_i (mM)	*Enzyme (µg)*	*Mg^{2+} (mM)*	*Rate (µmoles ^{14}C per mg chlorophyll per h)*	*% Stimulation (+) or inhibition (−)**
5 (control)	0	0	101	0
	21	0	126	+25
	0	1.0	115	+15
	21	1.0	138	+38
	0	2.0	110	+10
	21	2.0	17	−83
	0	5.0	48	−52
	21	5.0	2.3	−98
1 (control)	0	0	83	—
	8.4	0	158	+90
	0	1.0	115	+38
	8.4	1.0	108	+30
	0	1.5	93	+12
	8.4	1.5	3.5	−96

* Stimulation or inhibition is with respect to the controls with 5 mM PP_i or 1 mM PP_i, as indicated. Controls were chloroplasts suspended in Solution C *minus* Mg^{2+}, and with the amount of PP_i shown.

Analysis of purified Factor B, by atomic absorption spectroscopy showed that it contained approximately 10 % Mg^{2+}. In the presence of the usual suspending medium (—Mg^{2+}) which contained 5 mM PP_i, addition of 21 μg of purified fructose-1,6-diphosphatase caused 25 % stimulation in the rate of photosynthesis (Table I), whereas addition of enough Mg^{2+} to make its concentration 2 mM, caused a 10 % stimulation in the absence of added fructose-1,6-diphosphatase but an 85 % inhibition in the presence of the fructose-1,6-diphosphatase. Since the total volume was 0.5 ml, the added Mg^{2+} was 1 mM above the usual reaction mixture concentration and corresponded to 0.5 μmole, or 12 μg, of Mg^{2+}. This may be compared with the synergistic effect found previously[2] upon the addition of 24 μg of fructose-1,6-diphosphatase and 150 μg of Factor B, corresponding to 15 μg of Mg^{2+}.

At 1 mM PP_i (Table I) the inhibition by fructose-1,6-diphosphatase *plus* Mg^{2+} is even more striking. It should be particularly noted that whereas the stimulation with fructose-1,6-diphosphatase alone was 90 %, and the stimulation by 1.5 mM Mg^{2+} was 12 %, the addition of both factors caused an inhibition of 96 %.

When fructose-1,6-diphosphatase and Mg^{2+} were added to photosynthesizing chloroplasts after 6 min of photosynthesis with $H^{14}CO_3^-$, rather surprising effects on the levels of labeled metabolites occurred (Figs. 3 and 4). As compared with the levels of metabolites in the control chloroplasts, the level of Fru-1,6-P_2 in chloroplasts with added fructose-1,6-diphosphatase increased, while the level of Fru-6-*P* decreased. This behaviour has proved to be reproducible (6 experiments), as have other differences observed (Table II). These include an increased level of dihydroxyacetone-*P*, which is rapidly equilibrated with Fru-1,6-P_2 in all of our experiments, due to high aldolase activity both in the chloroplasts and in the medium. The kinetic curves for dihydroxyacetone-*P* are, in all cases, similar to the ones for Fru-1,6-P_2. Compounds with levels which decreased upon fructose-1,6-diphosphatase and Mg^{2+} addition included Glc-6-*P*, Sed-7-*P*, and Sed-1,6-P_2. The level of Sed-1,7-P_2 declined very rapidly upon fructose-

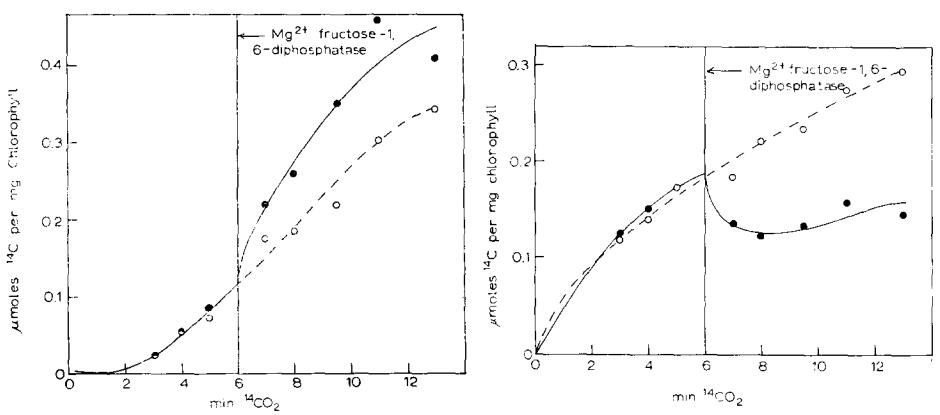

Fig. 3. Effect of addition of fructose-1,6-diphosphatase and Mg^{2+} on level of labeled Fru-1,6-P_2 in photosynthesizing spinach chloroplasts. ○, control (chloroplasts in Solution C); ●, 17 μg fructose-1,6-diphosphatase and Mg^{2+} to give 4 mM added after 6 min photosynthesis.

Fig. 4. Effect of addition of fructose-1,6-diphosphatase and Mg^{2+} on level of labeled Fru-6-*P* in photosynthesizing spinach chloroplasts. ○, control (chloroplasts in Solution C); ●, 17 μg fructose-1,6-diphosphatase and Mg^{2+} to give 4 mM Mg^{2+}.

TABLE II

EFFECT OF FRUCTOSE-1,6-DIPHOSPHATASE AND Mg^{2+} ON ^{14}C INCORPORATION INTO METABOLITES

Chloroplasts were suspended in Solution C *minus* Mg^{2+}.

Compound	*μmoles ^{14}C per mg chlorophyll*		
	After 5 min	*After 10 min*	
		Control	*4 min after addition of 4 mM Mg^{2+} and 16.8 μg fructose-1,6-diphosphatase*
Total ^{14}C uptake	3.65	9.00	6.68
3-*P*-Glycerate	1.38	3.18	2.39
Dihydroxyacetone-*P*	0.28	0.42	1.15
Fru-1,6-P_2	0.07	0.265	0.37
Fru-6-*P*	0.166	0.25	0.134
Glc-6-*P*	0.115	0.375	0.150
Sed-7-*P*	0.20	0.325	0.060
Sed-1,7-P_2	0.073	0.08	0.009
Pentose-*P*	0.070	0.22	0.22
Ribul-1,5-P_2	0.019	0.028	0.006
Origin	0.40	1.26	0.80
Miscellaneous spots	0.363	0.528	0.39
Total on chromatograms	3.14	6.93	5.68
% Accounted for	85.9	77.0	85.0
Photosynthesis rate (μmoles ^{14}C per mg chlorophyll per h)	43.8	54.0	40.1

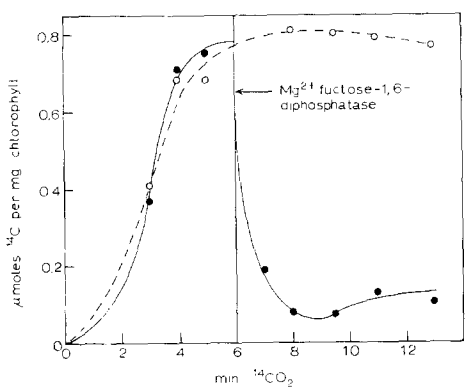

Fig. 5. Effect of addition of fructose-1,6-diphosphatase and Mg^{2+} on level of labeled Sed-1,7-P_2 in photosynthesizing spinach chloroplasts. ○, control (chloroplasts in Solution C); ●, 17 μg fructose-1,6-diphosphatase and Mg^{2+} to give 4 mM Mg^{2+}.

1,6-diphosphatase and Mg^{2+} addition (Fig. 5). The carboxylation substrate, Ribul-1,5-P_2 also decreased considerably, and this could be partly responsible for the decreased rate of CO_2 fixation. However, it should be noted that the levels of pentose monophosphate, which is the sum of Ribul-5-*P*, Xul-5-*P* and Rib-5-P, were not changed by the addition of Mg^{2+} and fructose-1,6-diphosphatase.

TABLE III

EFFECT OF ADDED Mg^{2+} AND FRUCTOSE-1,6-DIPHOSPHATASE ON DISTRIBUTION OF METABOLITES BETWEEN CHLOROPLASTS AND SUSPENDING MEDIUM

Photosynthesis rate was 78.0 μmoles ^{14}C per mg chlorophyll per h. "Washed chloroplasts", suspended in Solution C. Flask A: control, 1 mM Mg^{2+} and 5 mM PP_i. Flask B: same as flask A for 5.5 min of photosynthesis time; Mg^{2+} to make 6 mM and 10.2 μg fructose-1,6-diphosphatase added after 5.5 min.

Compound	*μmoles ^{14}C per mg chlorophyll*								
	*After 5 min**			*After 12 min including 6.5 min after Mg^{2+} and fructose-1,6-diphosphatase added to B*					
	Pellet	*Supernatant*	*Total*	*Pellet*		*Supernatant*		*Total*	
				A	*B*	*A*	*B*	*A*	*B*
Total ^{14}C incorporation			6.50					13.72	15.61
3-*P*-Glycerate	0.55	2.32	3.53	0.75	0.40	3.08	3.75	7.92	5.70
Dihydroxyacetone-*P*	0.04	0.73	0.95	0.04	0.03	0.60	2.45	0.74	3.01
Fru-1,6-P_2	0.02	0.19	0.27	0.01	0.01	0.11	1.32	0.19	1.49
Fru-6-*P*	0.094	0.024	0.186	0.202	0.054	0.054	0.110	0.303	0.184
Glc-6-*P*	0.240	0.040	0.275	0.529	0.095	0.080	0.136	0.588	0.280
Sed-7-*P*	0.36	0.03	0.034	0.715	0.185	0.090	0.075	0.675	0.190
Sed-1,7-P_2	0.015	0.012	0.135	0.014	0.004	0.005	0.029	0.115	0.066
Pentose-*P*	0.03	0.084	0.13	0.043	0.025	0.227	0.146	0.320	0.180
Ribul-1,5-P_2	0.016	0.005	0.046	0.038	0.013	0.008	0.109	0.078	0.166
Origin			0.80					1.73	1.55
Disaccharide	0.01	0.018	0.108	0.020	0.012	0.130	0.082	0.112	0.08
Glyceraldehyde-3-*P*	0.004	0.062	0.075	0.006	—	0.048	0.279	0.050	0.431
Miscellaneous								0.095	0.931
Total from chromatograms			6.76					12.92	14.26
% Accounted for			104.0					94.1	91.3

* Average of the levels found in Flasks A and B.

TABLE IV

EFFECT OF Mg^{2+} AND FRUCTOSE-1,6-DIPHOSPHATASE ADDED FROM THE BEGINNING OF PHOTOSYNTHESIS ON THE LEVEL OF METABOLITES SEEN AFTER 5 min WITH $^{14}CO_2$

Chloroplasts were suspended in Solution C (which contains 1 mM mg^{2+} and 5 mM PP_i); additional Mg^{2+} was added to give the final concentrations shown.

Compound	% *of total fixed*					
	Control			*21 μg fructose-1,6-diphosphatase*	*21 μg fructose-1,6-diphosphatase*	*21 μg fructose-1,6-diphosphatase*
	1 mM Mg^{2+}	*2 mM Mg^{2+}*	*3 mM Mg^{2+}*	*1 mM Mg^{2+}*	*2 mM Mg^{2+}*	*3 mM Mg^{2+}*
3-*P*-Glycerate	15	17	16	26	15	9.6
Dihydroxyacetone-*P*	28	27	29	26	35	39
Fru-6-*P*	2.3	2.0	1.9	1.6	1.6	1.4
Sed + Glc-*P*	5.3	4.8	4.3	4.0	3.8	3.8
Pentose-*P*	1.2	1.2	1.3	1.3	1.2	1.3
	μmoles ^{14}C per mg chlorophyll					
Total ^{14}C fixed	5.43	5.68	5.39	4.32	3.83	0.770

The effects of addition of fructose-1,6-diphosphatase and Mg^{2+} on the distribution of metabolites between the chloroplasts and the suspending medium are very large (Table III). In the chloroplasts with these additions the level of Fru-1,6-P_2 in the medium increased 11-fold, and the level of dihydroxyacetone-*P* increased 4-fold. At the same time, the level of Fru-6-*P* in the pellet declined 75 %, its level in the medium increased 2-fold, and its total level declined about 40 %. Thus, the addition of fructose-1,6-diphosphatase and Mg^{2+} has increased greatly the appearance of metabolites in the suspending medium. However, the added fructose-1,6-diphosphatase has not increased the conversion of Fru-1,6-P_2 to Fru-6-*P* in the total, although some increased conversion in the medium may have occurred.

When enough fructose-1,6-diphosphatase and Mg^{2+} are present from the start of photosynthesis to cause inhibition, the relative effects on the distribution of metabolites are similar to the effects when Mg^{2+} and fructose-1,6-diphosphatase are added after several minutes of photosynthesis (Table IV). The ^{14}C levels in all compounds are, of course, decreased, but relatively greater decreases are seen in hexose and heptose monophosphate compared with dihydroxyacetone-*P* which is proportional to Fru-1,6-P_2.

A comparison of $^{14}CO_2$ fixation rates in the presence of added fructose-1,6-diphosphatase and bovine serum albumin (Table V), each added with Mg^{2+}, shows that inhibition is not a general effect of protein addition.

TABLE V

COMPARISON OF EFFECTS OF ADDITION OF FRUCTOSE-1,6-DIPHOSPHATASE AND OF BOVINE SERUM ALBUMIN ON PHOTOSYNTHESIS RATE IN ISOLATED CHLOROPLASTS

5 min photosynthesis, 1 mM PP_i and 1.5 mM Mg^{2+}. Photosynthesis rate of control: 44.3 μmoles ^{14}C per mg chlorophyll per h.

μg protein per flask	*Relative rate of photosynthesis*	
	Fructose-1,6-diphosphatase	*Bovine serum albumin*
Control: "0"	1.0	1.0
8.4 μg	0.17	1.26

The effects of addition of fructose-1,6-diphosphatase alone, without enough Mg^{2+} to cause a synergistic effect in the presence of 5 mM PP_i, were minor (Table VI). There was a slight stimulation of $^{14}CO_2$ fixation rate, but levels of individual compounds in chloroplasts and medium were virtually unchanged.

The effects of addition of 5 mM Mg^{2+} alone, after 7 min photosynthesis in the normal medium (Solution C), were both qualitatively very similar to the effects of adding both fructose-1,6-diphosphatase and Mg^{2+} in one experiment (Table VII). However, the photosynthetic rate in this experiment with Mg^{2+} addition only was only 30 μmoles CO_2 fixed per mg chlorophyll per h, lower than usual, suggesting a greater than usual breakage of chloroplasts during the course of the experiment and, consequently, the presence of considerable fructose-1,6-diphosphatase in the medium, coming from broken chloroplasts. This sort of difficulty can never be entirely overcome under the conditions which we use, and it is probable that there is always present in

TABLE VI

EFFECT OF ADDED FRUCTOSE-1,6-DIPHOSPHATASE ON DISTRIBUTION OF METABOLITES BETWEEN CHLOROPLASTS AND SUSPENDING MEDIUM

Washed chloroplasts were suspended in Solution C (which contains 5 mM PP_i and 1 mM Mg^{2+}). Photosynthesis rate was 39.2 μmoles ^{14}C per mg chlorophyll per h. Flask A: control. Flask B: 30 μg fructose-1,6-diphosphatase added after 5.5 min photosynthesis.

Compound	*μmoles ^{14}C per mg chlorophyll*								
	After 5 min *			*After 12 min including fructose-1,6-diphosphatase in B for 6.5 min*					
	Pellet	*Supernatant*	*Total*	*Pellet*		*Supernatant*		*Total*	
				A	*B*	*A*	*B*	*A*	*B*
Total ^{14}C incorporated			3.27					6.82	7.55
3-*P*-Glycerate	0.35	1.09	1.52	0.460	0.460	2.28	2.64	2.49	3.16
Dihydroxyacetone-*P*	0.060	0.390	0.60	0.115	0.030	0.615	0.560	0.953	0.816
Fru-1,6-P_2	0.007	0.053	0.105	0.016	0.009	0.121	0.076	0.196	0.178
Fru-6-*P*	0.095	0.014	0.175	0.157	0.109	0.033	0.028	0.277	0.187
Glc-6-*P*	0.225	0.033	0.210	0.380	0.210	0.055	0.063	0.425	0.042
Sed-7-*P*	0.270	0.010	0.183	0.500	0.370	0.082	0.065	0.460	0.363
Sed-1,7-P_2	0.017	0.003	0.085	0.020	0.007	0.005	0.003	0.116	0.022
Pentose-*P*	0.025	0.085	0.125	0.058	0.041	0.245	0.320	0.380	0.392
Ribul-1,5-P_2	0.016	0.002	0.027	0.017	0.007	0.003	0.003	0.038	0.050
Miscellaneous spots, including origin			0.344					1.235	1.339
Total from chromatograms			3.37					6.57	6.93
% Accounted for			103.0					96.3	91.8

* Average of levels found in Flasks A and B.

the medium some fructose-1,6-diphosphatase, as well as all of the other enzymes from the chloroplasts.

In another experiment (Table IV) Mg^{2+} concentration was such that no inhibition was seen without added fructose-1,6-diphosphatase. In that case, no increased levels of metabolites were seen with Mg^{2+} without added fructose-1,6-diphosphatase.

As a consequence of chloroplast breakage, the medium in which the chloroplasts are suspended contains enzymes. Although many enzymes of the carbon reduction cycle retain some activity in the medium, we have found that there is no appreciable activity of either fructose-1,6-diphosphatase or ribulose diphosphate carboxylase (EC 4.1.1.39). When chloroplasts were placed in the light in Solution C for 5 min and centrifuged from the medium, assay of the medium for fructose-1,6-diphosphatase activity showed an activity of less than 0.1 nmole Fru-1,6-P_2 converted per min which may be compared with the CO_2 fixation rate of the chloroplasts of 20 nmoles per min, based on a rate of 60 μmoles/h per mg chlorophyll and 50 μg chlorophyll in the chloroplasts in the flask. Also, it was found that 10 μg of fructose-1,6-diphosphatase added gave an activity of only 0.2 nmole Fru-1,6-P_2 converted per min in Solution C, but had an activity of 100 nmoles/min at pH 8.7 and 5 mM Mg^{2+}.

When photosynthesis and metabolite transport of chloroplasts with 5 mM PP_i are compared with that of chloroplasts without PP_i (Solution C *minus* PP_i) ,the principal effects are seen to be the improved retention (with PP_i) in the chloroplasts of those metabolites which, under the usual conditions, tend to be retained, namely, hexose and heptose monophosphates and Ribul-1,5-P_2 (Table VIII).

TABLE VII

EFFECT OF ADDITION OF Mg^{2+} (5 mM) ON ^{14}C INCORPORATION INTO METABOLITES IN CHLOROPLASTS AND SUSPENDING MEDIUM

Photosynthesis rate was 33.2 μmoles ^{14}C per mg chlorophyll per h. Flask A: control; washed chloroplasts, suspended in Solution C (which contains 5 mM PP_i) *minus* Mg^{2+}. Flask B: same as control at start: 5 mM Mg^{2+} added after 7 min photosynthesis.

Compound	*μmoles ^{14}C per mg chlorophyll*								
	*After 6 min**			*After 15 min including 8 min after addition of 5 mM Mg^{2+} to Flask B*					
	Pellet	*Supernatant*	*Total*	*Pellet*		*Supernatant*		*Total*	
				A	*B*	*A*	*B*	*A*	*B*
Total ^{14}C incorporated			3.32					8.6	7.8
3-*P*-Glycerate	0.127	1.22	1.62	0.25	0.10	3.75	3.00	5.0	3.2
Dihydroxyacetone-*P*	0.033	0.48	0.58	0.03	0.01	0.27	0.59	0.41	0.90
Fru-1,6-P_2	0.01	0.18	0.18	0.012	0.005	0.09	0.70	0.15	0.85
Fru-6-*P*	0.055	0.009	0.078	0.076	0.022	0.028	0.12	0.11	0.14
Glc-6-*P*	0.132	0.008	0.111	0.154	0.05	0.05	0.076	0.227	0.171
Sed-7-*P*	0.158	0.006	0.095	0.288	0.05	0.043	0.065	0.244	0.14
Sed-1,7-P_2	0.015	0.008	0.087	0.021	0.003	0.005	0.019	0.100	0.03
Pentose-*P*	0.021	0.048	0.073	0.025	0.008	0.13	0.165	0.17	0.19
Ribul-1,5-P_2	0.007	0.001	0.017	0.02	0.006	0.003	0.002	0.04	0.02
Origin			0.46					1.41	1.21
Miscellaneous			0.83					0.556	0.56
Total from chromatograms			3.38					8.42	7.45
% Accounted for			102					97.9	95.4

* Average of levels found in Flasks A and B.

Additions of low concentrations of Fru-1,6-P_2 to chloroplast suspensions (Table IX) before the start of photosynthesis result in some stimulation. Larger concentrations of added Fru-1,6-P_2 produce inhibition of CO_2 uptake. These effects also depend somewhat on the concentration of added PP_i, the stimulation being somewhat more pronounced with 1 mM PP_i than with 5 mM PP_i whereas the inhibition is greater in the presence of 5 mM PP_i.

Addition of 1 mM Fru-1,6-P_2 to chloroplasts after 5 min of photosynthesis with $^{14}CO_2$ (Table X) causes inhibition (1.9 μmoles $^{14}CO_2$ fixed per mg chlorophyll during the next 6 min compared with 6.7 μmoles fixed in the control and 7.7 μmoles fixed in the presence of added Fru-6-*P*). The effects of added Fru-6-*P* are rather minor, suggesting little penetration of this compound into the chloroplasts. The effects that are seen with added Fru-1,6-P_2 (note the "flushing" of the ^{14}C from the Fru-6-*P* and other subsequent pools) suggest that the Fru-1,6-P_2 does enter the chloroplasts readily.

DISCUSSION

The principal regulatory effects on the rate of photosynthesis by isolated chloroplasts previously ascribed to a substance of high molecular weight *plus* a substance of low molecular weight in the spinach juice[1] can be completely duplicated by the addition of appropriate amounts of fructose-1,6-diphosphatase and of Mg^{2+}. Fru-1,6-P_2 comes out rapidly from the isolated photosynthesizing spinach chloroplasts, while

TABLE VIII

EFFECT OF PP_i ON ^{14}C INCORPORATION INTO METABOLITES IN CHLOROPLASTS AND SUSPENDING MEDIUM

Chloroplasts were suspended in Solution C (which contains 5 mM PP_i) or in Solution C *minus* PP_i, as indicated.

Compound	*μmoles ^{14}C per mg chlorophyll*											
	After 6 min						*After 15 min*					
	Pellet		*Supernatant*		*Total*		*Pellet*		*Supernatant*		*Total*	
	$+PP_i$	$-PP_i$	$+PP_i$	$-PP_i$	$+PP_i$	$-PP_i$	$+PP_i$	$-PP_i$	$+PP_i$	$-PP_i$	$+PP_i$	$-PP_i$
Total ^{14}C incorporated					6.1	3.92					9.1	6.1
3-*P*-Glycerate	0.45	0.27	1.19	1.15	1.41	1.22	0.84	0.33	1.63	1.29	1.77	1.60
Dihydroxyacetone-*P*	0.013	0.103	0.50	0.35	0.32	0.19	0.01	0.001	0.150	0.045	0.10	0.013
Fru-1,6-P_2	0.010	0.008	0.215	0.086	0.275	0.162	0.003	0.001	0.020	0.001	0.05	0.005
Fru-6-*P*	0.102	0.043	0.018	0.035	0.165	0.090	0.145	0.020	0.028	0.035	0.198	0.090
Glc-6-*P*	0.282	0.10	0.030	0.020	0.290	0.133	0.40	0.050	0.067	0.073	0.494	0.193
Sed-7-*P*	0.390	0.145	0.022	0.010	0.261	0.125	0.34	0.090	0.085	0.022	0.44	0.135
Sed-1,7-P_2	0.006	0.001	0.007	0.012	0.033	0.024	0.003	—	0.002	0.001	0.015	0.003
Pentose-*P*	0.017	0.007	0.080	0.050	0.129	0.064	0.030	0.008	0.123	0.024	0.181	0.053
Ribul-1,5-P_2	0.016	0.010	0.002	0.002	0.029	0.018	0.037	0.010	0.004	0.002	0.054	0.020
Phosphoenolpyruvate	—	—	0.002	0.004	0.002	0.005	—	0.0014	0.0024	0.013	0.003	0.023
Origin					0.717	0.672					1.08	0.95
Disaccharide	0.014	0.010	0.028	0.023	0.036	0.028	0.037	0.022	0.180	0.127	0.201	0.206
Miscellaneous					0.267	0.193					1.012	0.922
Total on chromatograms					3.934	2.924					5.598	4.213
% Accounted for					64.5	74.6					61.5	69.1
Photosynthesis rate (μmoles per mg chlorophyll per h)					61.0	39.2						

TABLE IX

EFFECT OF CONCENTRATION OF ADDED Fru-1,6-P_2 ON $^{14}CO_2$ UPTAKE DURING 5 min PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS

Chloroplasts were suspended in Solution C (which contains 5 mM PP_i) or in Solution C except that PP_i was 1 mM, as indicated. Photosynthesis rates (no Fru-1,6-P_2 added) were 43.4 and 53.8 μmoles ^{14}C per mg chlorophyll per h with 1.0 and 5.0 mM PP_i, respectively.

Fru-1,6-P_2 Concn. (mM)	*Fixation of ^{14}C (μmoles per mg chlorophyll)*	
	1 mM PP_i	*5 mM PP_i*
0	3.6	4.5
0.1	5.5	5.3
0.2	6.4	5.6
0.5	5.3	3.8
1.0	3.7	1.9

TABLE X

EFFECT OF ADDED Fru-1,6-P_2 AND OF Fru-6-P ON PHOTOSYNTHESIS RATE AND INCORPORATION OF ^{14}C INTO METABOLITES

Photosynthesis rate was 97.2 μmoles ^{14}C per mg chlorophyll per h. Chloroplasts were suspended in Solution C.

Compound	*μmoles of ^{14}C per mg chlorophyll*			
	After 5 min, no additions	*After 11 min*		
		Control	*6 min after addition of 1 mM Fru-6-P*	*6 min after addition of 1 mM Fru-1,6-P_2*
Total ^{14}C incorporated	8.1	14.8	15.8	10.0
3-*P*-Glycerate	2.65	4.75	4.60	2.70
Dihydroxyacetone-*P*	0.93	1.19	1.27	1.38
Fru-1,6-P_2	0.23	0.74	0.50	1.15
Fru-6-*P*	0.205	0.26	0.37	0.05
Glc-6-*P*	0.225	0.435	0.425	0.10
Sed-7-*P*	0.235	0.25	0.35	0.045
Sed-1,7-P_2	0.115	0.145	0.460	0.075
Pentose-*P*	0.120	0.180	0.66	0.11
Ribul-1,5-P_2	0.035	0.064	0.062	0.01
Origin	0.70	1.92	2.00	0.85
Miscellaneous	0.425	0.896	1.028	0.81
Total on chromatogram	5.87	10.83	11.73	7.28
% Accounted for	72.5	73.2	76.6	72.8

Fru-6-*P* is well retained[13]. It might have been postulated that added fructose-1,6-diphosphatase converts Fru-1,6-P_2 to Fru-6-*P* which cannot re-enter the chloroplast. This would remove carbon from the photosynthetic carbon reduction cycle, leading to a shortage of carboxylation substrate, Ribul-1,5-P_2. The fact that PP_i is an inhibitor of fructose-1,6-diphosphatase[16] while Mg^{2+} is an activator of this enzyme, would be consistent with this assumption.

However, the data presented force the conclusion that the primary effect of

added fructose-1,6-diphosphatase on photosynthesis by isolated chloroplasts is not produced by means of its function in converting Fru-1,6-P_2 to Fru-6-P. (1) Fructose-1,6-diphosphatase is nearly inactive under the conditions that exist in the suspending medium of the chloroplasts. (2) The level of labeled Fru-1,6-P_2 increases and the level of labeled Fru-6-P decreases on addition of the fructose-1,6-diphosphatase. (3) Addition of fructose-1,6-diphosphatase leads to lower levels of hexose and heptose monophosphates and of Ribul-1,5-P_2 within the pellet and to an increased level of Fru-1,6-P_2 in the supernatant solution. Similar effects are seen with added Mg^{2+} and the effects of Mg^{2+} and fructose-1,6-diphosphatase are synergistic.

The ratio of the volume of the supernatant solution to the chloroplast volume is of the order of 50:1. Thus, even for those metabolites which rapidly appear in the suspending medium, active transport is not required. Nevertheless, given the very considerable differences in rates at which the various metabolites appear in the medium (*e.g.*, Fru-1,6-P_2 *vs.* Fru-6-P), it seems clear that some kind of facilitated transport is required. We conclude that the movement of Fru-1,6-P_2 and of dihydroxyacetone phosphate from the chloroplast to the suspending medium is enhanced by the addition of fructose-1,6-diphosphatase and Mg^{2+} to the medium. We tentatively conclude that fructose-1,6-diphosphatase or some compound derived from it is capable of being absorbed somewhere in the outer chloroplast double membrane and there affects metabolite diffusion rates. Such activity apparently would be stimulated by Mg^{2+} and inhibited by PP_i. Studies of metabolite transport and other physical and chemical properties of the chloroplast membrane probably will be required before such a postulate can be proved or disproved.

The effect of added PP_i is to increase the levels of hexose and heptose monophosphates and of Ribul-1,5-P_2 within the chloroplasts and to diminish their appearance in the supernatant solution. However, the levels of Fru-1,6-P_2 and of dihydroxyacetone-P appearing and remaining in the supernatant solution are greater in the presence of PP_i than in its absence. This may be due to the higher rate of photosynthesis and larger amount of reduced carbon when PP_i is present.

It now appears that only a few steps in the photosynthetic carbon reduction cycle are necessarily restricted to the inside of the chloroplasts in the suspension of isolated chloroplasts which unavoidably contains broken chloroplasts and soluble enzymes in the medium. The steps which apparently occur exclusively inside the chloroplasts are, as might be expected, the rate-limiting steps or regulated steps, that is, the diphosphatase and the carboxylation reactions. Reactions mediated by aldolase, transketolase and isomerases appear to occur to some extent outside the chloroplasts. Probably the reduction of 3-P-glycerate to triose-P and the phosphorylation of Rib-5-P to Rib-1,5-P_2 also occur almost entirely in the chloroplasts.

It was found in earlier studies[13] and again in the present investigation (see Table VIII) that levels of Fru-1,6-P_2 and dihydroxyacetone-P in the supernatant solution rise very high by about 6–10 min, and then decline between 10 and 20 min of photosynthesis in isolated chloroplasts. Apparently, the longer the concentration of these metabolites stays above a low level, the longer the rate of CO_2 uptake is maintained at a high level. It was found earlier that addition of Fru-1,6-P_2 to the chloroplasts tends to maintain a high rate at a time when the rate would decline. The finding that small amounts of Fru-1,6-P_2 stimulates fixation of CO_2 while larger amounts inhibit provides further evidence for the regulatory effects of Fru-1,6-P_2.

The simplest explanation of the regulatory effects on CO_2 fixation rate of fructose-1,6-diphosphatase, Mg^{2+} and PP_i is based on the respective effects on these factors on the movement of metabolites out from the chloroplasts into the suspending medium. The retention of hexose and heptose monophosphates, and especially of Ribul-1,5-P_2 in the chloroplasts, probably helps to maintain CO_2 fixation. In addition, it is conceivable that there may be a direct regulatory effect of Fru-1,6-P_2 concentration on the rate of the carboxylation reaction in whole chloroplasts. The mechanism of such a regulation might operate either on the carboxylation enzyme or on the CO_2 transport into the chloroplasts.

It seems possible that fructose-1,6-diphosphatase in the outer chloroplast membrane might play an important role in regulation of photosynthetic metabolism *in vivo*. The postulated role would be in the movement of carbon compounds from the carbon reduction cycle in the chloroplast to other types of metabolism in the cytoplasm. The control of this "transport" activity could be largely mediated by Mg^{2+} and PP_i concentration inside and outside the chloroplast outer membrane.

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We have found recently that we can obtain from the fructose diphosphatase, upon denaturation, a heat-stable factor which induces, although at a diminished activity, some of the effects on CO_2 fixation rates seen with the active enzyme. However, these effects do not exhibit the strong synergism with Mg^{2+} ion found with the active enzyme.

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