Plant Physiol. (1967) 42, 1321–1328

Chloroplast Integrity and ATP-Dependent CO_2 Fixation in Spinacia oleracea

Emmett J. Johnson[1] and Barbara S. Bruff[2]

Exobiology Division, Ames Research Center, National Aeronautics and Space Administration, Moffett Field, California 94035

Received March 15, 1967.

Summary. Washed whole chloroplasts of *Spinacia oleracea* isolated and assayed in a tris (hydroxymethyl aminomethane)-HCl buffered sucrose solution exhibited low dark CO_2 fixing activity, whereas washed whole chloroplasts isolated in the same buffer but assayed in that buffer without sucrose exhibited much greater dark CO_2 fixing activity. The lowered activity could be attributed to the impermeability of the chloroplast membrane to ribose-5-phosphate or adenosine triphosphate. The preservation of the integrity of the chloroplast membrane, as reflected by its impermeability to either or both of the abovementioned compounds, was measured by the fixation of $^{14}CO_2$ into acid-stable products in the presence of ribose-5-phosphate and adenosine triphosphate by the whole chloroplast as compared with fixation by the chloroplast extract. An effect (i.e., apparent resistance to the passage of ribose-5-phosphate or adenosine-5-triphosphate into the chloroplast) similar to, but less pronounced than, that produced by the presence of sucrose in the isolation medium was observed upon the addition of $MnCl_2$ or $CaCl_2$ to the buffered sucrose isolation medium. The addition of KCl enhanced slightly the effect produced by addition of sucrose alone to the isolation medium. The presence of $MgCl_2$ in the isolation medium, however, either caused the chloroplasts to become leaky or more fragile since more of the activity of the carboxylative phase enzymes appeared in the cytoplasm. When a mixture of all of the metal ions was added to the buffered sucrose suspending medium, the chloroplasts exhibited the same response observed with $MgCl_2$ alone. The addition of ethylene diaminetetraacetate or dithiothreitol appeared to alter the permeability of the chloroplast membrane nonspecifically when the assay was conducted in the absence of sucrose. Specific activities (μmoles CO_2 fixed/mg chlorophyll $\times$ hr) as high as 329.6 have been observed for dark fixation by chloroplasts. The phosphoenolpyruvate carboxylase activity in the chloroplasts was only one-seventh that of ribulose diphosphate carboxylase. The phosphoenolpyruvate carboxylase activity in the cytoplasm was 5 times that of the chloroplasts.

Although considerable progress has been made in the study of photosynthesis, the relationship of light-dependent ATP and NADPH synthesis to ATP- and NADPH-dependent CO_2 fixation by green plants remains unresolved. One point of view is that the sole function of light in the photosynthetic process is the generation of ATP and reduction of pyridine nucleotide (2, 9). Another point of view is that ATP and NADPH alone are insufficient to promote dark CO_2 incorporation into carbohydrates (13). The primary difficulty in arriving at firm conclusions concerning CO_2 fixation when light is the source of ATP and ribose-5-P is produced endogenously, in contrast to CO_2 fixation when ATP and ribose-5-P are provided exogenously, has been the low levels of activity observed under either condition as compared with the highest reported values for the intact leaf (17, 18, 23). In the investigations from which the available information has been derived, a wide variety of isolation and assay procedures has been employed. There is no assurance that any given isolation or assay technique which allows good rates of photophosphorylation or of CO_2 fixation independently, will support maximum rates of these 2 activities simultaneously. In most of the investigations of light-dependent CO_2 fixation either the potential of the carboxylative phase enzymes was not measured (3, 17, 23, 24) by the addition of ATP and ribose-5-P, or the activity in the presence of added ATP was lower than the light-dependent activity (13). Although light-dependent ATP synthesis has reached levels of over 300 μmoles/mg chlorophyll $\times$ hour in

[1] Present address: Bruce Lyon Memorial Research Laboratory, 51st and Grove Streets, Oakland, California.

[2] Present address: Department of Marine Biology, University of California, Scripps Institution of Oceanography, La Jolla, California.

isolated spinach chloroplasts (10) and over 1000 μmoles/mg chlorophyll × hour in isolated Swiss chard chloroplasts (4, 11), the fixation of $^{14}CO_2$ in the light or in the presence of ATP has not exceeded 155 μmoles/mg chlorophyll × hour (17), and usually has been considerably less than the maximum fixation rate of approximately 245 μmoles/mg chlorophyll × hour achieved by the intact leaf (17). Investigations on the influence of cofactors on ATP-dependent CO_2 fixation in cell-free systems from photosynthetic and chemosynthetic autotrophs (12) revealed that certain ratios of the various components were essential for obtaining maximum ATP-dependent CO_2 fixation. As a result of these findings, specific activities for ATP-dependent CO_2 fixation could be obtained which were highest yet reported. The use of this assay system has allowed an investigation of the relation of chloroplast integrity to ATP-dependent CO_2 fixation, and has provided information concerning the permeability of the chloroplast membrane to ATP and ribose-5-P, as well as the relationship between ribose-5-P and ATP-dependent and ribulose-1, 5-diP dependent CO_2 fixation in various fractions from spinach leaves. Additionally, information on the effect of EDTA and sulfhydryl reagents on intact chloroplasts, as well as on soluble enzymes for CO_2 fixation, has been obtained.

Materials and Methods

Isolation and Fractionation of Spinach Leaf Homogenates. Fresh spinach leaves (*Spinacia oleracea*) were removed from plants grown in a greenhouse at 15° and 60% relative humidity. Chloroplasts prepared from spinach obtained from local markets or spinach picked on a farm within 5 minutes driving time from the laboratory had good CO_2 fixing ability, but chloroplasts from the market spinach were completely permeable to ribose-5-P and ATP immediately after preparation.

All steps in the processing were carried out at 2° to 4°. The midribs and petioles were removed and only laminae were retained. All glassware and other apparatus were precooled and kept in crushed ice or ice water near 0°. The laminae were washed 2 or 3 times in cold distilled water (2°-4°). The washed laminae were blotted dry with cheesecloth. Routinely, 50 g of washed and dried laminae were placed in a Waring Blendor jar with 100 ml of tris buffered sucrose suspending medium (0.1 M tris-HCl buffer, pH 7.5, containing 0.5 M sucrose). In some experiments, specific salts were added to the buffered suspending medium and these will be described in the text dealing with the data for those experiments. The leaf suspension was homogenized for exactly 10 seconds at full speed. The homogenate was then passed through 4 thicknesses of cheesecloth which had been washed with distilled water. The material passing through the cheesecloth was processed according to the flow diagram presented in figure 1.

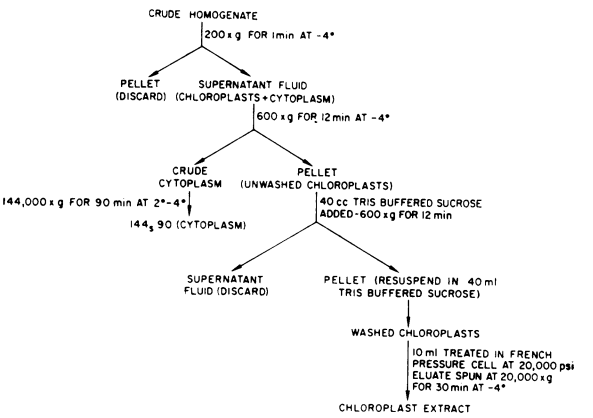

FIG. 1. Procedure for fractionation of leaf homogenates of *Spinacia oleracea*.

Protein was determined according to the method of Lowry et al. (19) and chlorophyll according to the method of Arnon (1).

Assay for $^{14}CO_2$ Fixation. The incorporation of CO_2 (as $Na_2{}^{14}CO_3$) into acid-stable products was conducted in double sidearm 20-ml Warburg flasks. The main compartment contained, in a total volume of 2.7 ml, in μmoles: tris buffer, pH 7.5, 100; ribose-5-P, 10; ATP, 20; $MgCl_2$, 160; and $Na_2{}^{14}CO_3$, 30 (2.5 × 10^5 cpm/μmole). Whenever EDTA was present the final concentration was 5 × 10^{-4} M. The fraction to be assayed was placed in 1 sidearm and 0.3 ml of 10% perchloric acid in the other. One sidearm was plugged with a sidearm plug with a gassing vent and the other was sealed with a serum cap. The flasks were placed on manometers and into a Warburg bath at 30°. The flasks were gassed with helium for 5 minutes, the fraction to be assayed was tipped in from the sidearm, and the reaction allowed to take place for 10 minutes with shaking. At the end of this time, the perchloric acid was tipped in from the second sidearm to terminate the reaction and to release any unfixed $^{14}CO_2$. Then 0.1 ml of 30% NaOH was injected into the sidearm. The flasks were then shaken for an additional 20 minutes.

Determination of Fixed $^{14}CO_2$. The contents of the Warburg flasks were centrifuged to remove precipitated protein. Two-tenths ml of the supernatant fluid was placed in a counting vial containing 10 ml of scintillation fluid (2,5-diphenyloxazole, 4 g; 1,4-*bis*-2′[5′-phenyloxazolyl] benzene, 100 mg; toluene, 700 ml; absolute ethanol, 300 ml) and counted in a Packard scintillation counter.

Chemicals. All chemicals in this investigation were purchased from commercial sources and were reagent grade. The ribose-5-P, ATP, and P-enolpyruvate were purchased either from Calbiochem or Sigma Chemical Company. When necessary, solutions were neutralized before use. The ribulose-1, 5-diP was obtained from Sigma Chemical Company. The barium salt was converted to sodium salt by treatment with Dowex 50 (H^+) to remove the barium

and neutralized with NaOH. $Na_2{}^{14}CO_3$ was purchased either from Volk Chemical Company or New England Nuclear Corporation.

Results and Discussion

Chloroplast Permeability and Dark CO_2 Fixation. The determination of the permeability or impermeability of the chloroplast membrane was based on a physiological measurement. The CO_2 fixation process is dependent on ATP and ribose-5-P as well as Mg^{2+}. If whole chloroplasts showed little or no activity in the presence of ribose-5-P and ATP but an extract of the same chloroplasts showed good activity, the semipermeability property of the chloroplast membrane was assumed to have been preserved. Chloroplasts isolated in tris-HCl buffer made 0.5 M with respect to sucrose, and assayed in the same buffer, were impermeable to ribose-5-P or ATP. Chloroplasts isolated in tris-HCl buffer made 0.5 M with respect to sucrose, and assayed in tris-HCl buffer without sucrose, were permeable to ribose-5-P and ATP.

The CO_2 fixing activity of washed whole chloroplasts isolated and assayed in tris-sucrose was lower than that of washed whole chloroplasts isolated in tris-sucrose, but assayed in tris (table I). This suggests that the chloroplasts isolated and assayed in tris-sucrose were less permeable to externally provided ribose-5-P or ATP. That the retention of chloroplast membrane integrity and not an inhibition of the carboxylative phase enzymes accounts for the lowered activity of tris-sucrose isolated and assayed chloroplasts can be seen in table II. Extracts of these chloroplasts had considerable enzymatic activity, and sucrose did not inhibit appreciably this activity. This is best seen when dithiothreitol is present, because then maximum activity is observed. The presence of dithiothreitol or EDTA resulted in retention of greater activity in these extracts. Adding both EDTA and dithiothreitol simultaneously had the same effect as adding either of them alone, suggesting that they have equivalent, but not additive, effects. The crude cytoplasm centrifuged at 144,000 $\times$ *g* for 90 minutes (144_s90) had considerable activity, suggesting that the isolation procedure did result in the breakage of some chloroplasts (table III). This was confirmed by the presence of chlorophyll in this fraction before the high-speed centrifugation.

It appears from these results that chloroplasts isolated and assayed in the sucrose medium were impermeable to either ribose-5-P or ATP. The exclusion of either or both of these components would produce the observed results. There is evidence in the literature that intact chloroplasts are permeable to ATP and many intermediates of the carbon reduction cycle (6, 7, 8, 14, 15, 16, 21, 22). One possible explanation of these differences may be the differences in the source and treatment of the respective plant material. In our hands the permeability properties described were exhibited to the greatest degree only by chloroplasts isolated from leaves which were excised in the greenhouse and immersed immediately in distilled water at 2° to 4°. Freshly picked farm-grown spinach showed a low degree of permeability, with some variability from sample to sample. Chloroplasts from spinach obtained from local markets were completely permeable to ribose-5-PO_4 and ATP

Table I. *The Effect of the Addition of Mg^{2+} to the Buffered Suspending Medium on ATP-Dependent CO_2 Fixation in Washed Whole Chloroplasts*

The complete system contained in μmoles: tris-HCl buffer, pH 7.5, 100; ribose-5-P, 10; ATP, 20; $MgCl_2$, 160; and $Na_2{}^{14}CO_3$, 30 (2.5×10^5 cpm/μmole). Total volume was 2.7 ml. Incubation time was 10 min at 30° in an atmosphere of helium. Dithiothreitol, 0.001 M and EDTA 5×10^{-4} M, final concentration. The data presented in tables I, II, and III were from a single experiment performed on the same day. The experiment was repeated at least 3 times.

System	Chloroplasts isolated without Mg^{2+}			Chloroplasts isolated with Mg^{2+}		
	CO_2 fixed	Specific activity*	Specific activity**	CO_2 fixed	Specific activity*	Specific activity**
Washed chloroplasts						
Assayed in tris buffer (—sucrose)	μmoles			μmoles		
Complete	1.17	12.6	123.0	0.41	7.2	70.2
— ATP	0.09	1.2		0.06	1.2	
— Ribose-5-P	0.09	1.2		0.08	1.2	
— Mg^{2+}	0.07	0.6		0.08	1.2	
+ EDTA	3.13	33.6	329.6	1.09	19.2	
Assayed in tris buffer (+sucrose)						
Complete	0.12	1.2		0.18	3.0	
— ATP	0.12	1.2		0.09	1.8	
— Ribose-5-P	0.14	1.8		0.05	1.2	
— Mg^{2+}	0.10	1.2		0.08	1.2	
+ EDTA	0.27	3.0		0.16	3.0	

* Specific activity is reported as μmoles of CO_2 fixed/mg protein $\times$ hour.
** Specific activity is reported as μmoles of CO_2 fixed/mg chlorophyll $\times$ hour.

Table II. *The Effect of the Addition of Mg^{2+} to the Buffered Suspending Medium on ATP-Dependent CO_2 Fixation in Chloroplast Extracts*

The complete system contained in μmoles: tris-HCl buffer, pH 7.5, 100; R-5-P, 10; ATP, 20; $MgCl_2$, 160; and $Na_2{}^{14}CO_3$, 30 (2.5×10^5 cpm/μmole). Total volume was 2.7 ml. Incubation time was 10 min at 30° in an atmosphere of helium. Dithiothreitol, 0.001 M final concentration.

System	Fraction isolated without Mg^{2+}		Fraction isolated with Mg^{2+}	
	CO_2 fixed	Specific activity*	CO_2 fixed	Specific activity*
Chloroplast extract				
Assayed in tris buffer (—sucrose)				
	μmoles		μmoles	
Complete	1.11	16.2	0.34	10.8
— ATP	0.09	1.2	0.10	3.0
— Ribose-5-P	0.41	0.6	0.18	5.4
— Mg^{2+}	0.10	1.2	0.06	1.8
+ EDTA	2.62	38.4	1.11	35.4
Assayed in tris buffer (+sucrose)				
Complete	0.63	9.0	0.17	5.4
+ EDTA	2.00	29.4	0.78	24.6
Chloroplast extract + DTT				
Assayed in tris buffer (—sucrose)				
	μmoles		μmoles	
Complete	2.51	33.0	1.36	37.2
— ATP	0.03	0.6	0.03	0.6
— Ribose-5-P	0.02	0.6	0.03	0.6
— Mg^{2+}	0.02	0.6	0.04	1.2
+ EDTA	2.66	34.8	1.48	40.2
Assayed in tris buffer (+sucrose)				
Complete	2.16	28.2	1.13	31.2
+ EDTA	2.34	30.6	1.28	34.8

* As defined in table I.

Table III. *The Effect of the Addition of Mg^{2+} to the Buffered Suspending Medium on ATP-Dependent CO_2 Fixation in the Cytoplasm*

The complete system contained in μmoles: tris-HCl buffer, pH 7.5, 100; R-5-P, 10; ATP, 20; $MgCl_2$, 160; and $Na_2{}^{14}CO_3$, 30 (2.5×10^5 cpm/μmole). Total volume was 2.7 ml. Incubation time was 10 min at 30° in an atmosphere of helium. Dithiothreitol, 0.001 M final concentration.

System	Fraction isolated without Mg^{2+}		Fraction isolated with Mg^{2+}	
	CO_2 fixed	Specific activity*	CO_2 fixed	Specific activity*
144_s90 (cytoplasm)				
Assayed in tris buffer (—sucrose)				
	μmoles		μmoles	
Complete	2.08	18.0	2.69	22.2
— ATP	0.04	0.6	0.05	0.6
— Ribose-5-P	0.05	0.6	0.05	0.6
— Mg^{2+}	0.03	0.6	0.08	0.6
+ EDTA	6.75	58.8	7.28	60.6
Assayed in tris buffer (+sucrose)				
Complete	1.72	15.0	1.65	13.8
+ EDTA	1.44	12.6	6.16	51.6
144_s90 (cytoplasm) + DTT				
Assayed in tris buffer (—sucrose)				
	μmoles		μmoles	
Complete	7.95	59.4	6.49	51.6
— ATP	0.05	0.6	0.10	0.6
— Ribose-5-P	0.07	0.6	0.10	0.6
— Mg^{2+}	0.06	0.6	0.12	1.2
+ EDTA	8.13	61.2	7.58	60.6
Assayed in tris buffer (+sucrose)				
Complete	5.73	43.2	5.46	43.8
+ EDTA	6.83	51.0	6.46	51.6

* As defined in table I.

immediately after preparation. This suggests that the chloroplast membrane undergoes a change with respect to its permeability properties proportional to the length of time the plant has been removed from its natural environment.

Only dark CO_2 fixation was measured in these studies while the other studies dealing with permeability dealt primarily with light-dependent CO_2 fixation. It is possible that some of the differences may be attributed to differences in chloroplasts carrying out light dependent CO_2 fixation and those carrying out dark CO_2 fixation.

Metal Ion Content of the Isolation Medium and Dark CO_2 Fixation. Table I shows the results of experiments in which the effect of adding $MgCl_2$ to the tris buffered sucrose solution used for chloroplast isolation was determined. Washed whole chloroplasts isolated in the above medium without $MgCl_2$ and assayed in tris buffer without sucrose fixed considerable $^{14}CO_2$ (120 μmole/mg chlorophyll $\times$ hr) in the presence of ribose-5-P and ATP, but not in their absence. This shows that the permeability of the membranes of these whole chloroplasts had been sufficiently altered to allow passage of externally added ATP and ribose-5-P into the chloroplasts. When $MgCl_2$ was added to the isolation medium, the observed specific activity (protein) was lowered to about half that observed in washed whole chloroplasts isolated in tris-sucrose without added $MgCl_2$. At the same time the activity in the chloroplast extract was lower in the presence of $MgCl_2$ than in its absence (table II), but the activity in the cytoplasm (table III) was greater when $MgCl_2$ was present in the isolation medium than when it was absent. This suggests that the presence of $MgCl_2$ makes the chloroplast membrane leaky and thus enzyme protein leaks out into the cytoplasm. Alternatively, the chloroplasts may become more fragile and break, releasing their enzymes to the cytoplasm. The sum total of the activities of the chloroplast (or chloroplast extract) and the cytoplasm are equal in the respective cases of the presence or absence of $MgCl_2$ in the isolation medium.

When EDTA was added to the assay medium containing chloroplasts isolated by both techniques (with and without Mg^{2+}), but assayed in tris without sucrose, the activity increased considerably over the respective controls without EDTA (table I). The interpretation of these data was complicated by the fact that EDTA also stabilizes or stimulates the carboxylative phase enzymes in the chloroplast extract (table II). However, this does not alter the fact that in the presence of EDTA, but not in its absence, ribose-5-P and ATP appeared to enter the chloroplast (table I). This suggests that in the absence of sucrose in the assay medium, the permeability of the chloroplast membrane was altered by EDTA.

The specific activity of the carboxylative phase enzymes in these chloroplasts was very high, as much as 329.6 μmoles of CO_2 fixed/mg chlorophyll $\times$ hour in the presence of EDTA (table I). The high specific activity is partly a result of incubating the systems at 30° as compared to 17° to 20° used by most other workers. However, as pointed out by Baldry et al. (5) the differences in increases in rates between 10° and 20° are larger than those between 20° and 30°. We chose 30° as the temperature of incubation because other work to be published later showed that this was the optimum temperature for the carboxylative phase enzymes.

In the presence of Mg^{2+} there was an aggregation in these extracts of chlorophyll-containing particles which are sedimented by the 20,000-g spin routinely used to clear the extract. Consequently, the 20,000-g supernatant fluid contained less chlorophyll without affecting the activity or concentration of the carboxylative phase enzymes. This aggregation of particles occurred only with Mg^{2+} and not with any of the other cations tested (which included all the cations reported in the literature as having been employed in the isolation of spinach chloroplasts). Consequently, the specific activities for the chloroplast extract are given per mg protein only.

The experiments reported with magnesium ions were repeated with K^+, Ca^{2+}, and Mn^{2+} separately, and with a mixture of Mg^{2+}, K^+, Ca^{2+}, and Mn^{2+}. In no instance (except for K^+ and then only slightly)

Table IV. *Comparison of Methods of Isolation and Assay for the Measurement of ATP-Dependent CO_2 Fixation*

In procedure A, the isolation solution contained the following: 0.33 M sorbitol; Na_2HPO_4-KH_2PO_4 buffer (0.1 M), pH 6.8; NaCl (0.1 %); $MgSO_4$ (0.1 %); and sodium isoascorbate (0.1 %). Assay solution contained the following: sorbitol (0.11 mM), $MgSO_4$ (0.8 mM), $MnCl_2$ (0.8 mM), KH_2PO_4 (0.8 mM), NaCl (0.08 mM), tris (0.25 mM), EDTA (0.8 mM), sodium isoaccorbate (1.6 mM), cysteine hydrochloride (1.6 mM) and 5 μmoles of Na_2CO_3 (2.5×10^5 cpm/μmole). The pH was 7.5 and the total volume was 2.7 ml (Walker-24). To all flasks ATP, 20 μmoles and R-5-P, 10 μmoles were added. In procedure B, isolation and assay techniques as described in Methods section of this paper.

Methods of isolation and assay	Specific activity
Washed chloroplasts	μmoles CO_2 fixed min^{-1} mg $protein^{-1}$
Isolation A, Assay A	0.2
Isolation A, Assay B	11.3
Isolation B, Assay A	0.1
Isolation B, Assay B	15.2
Chloroplast extract	
Isolation A, Assay A	0.1
Isolation A, Assay B	22.0
Isolation B, Assay A	0.0
Isolation B, Assay B	29.8
144_s90 (cytoplasm)	
Isolation A, Assay A	0.0
Isolation A, Assay B	25.4
Isolation B, Assay A	0.0
Isolation B, Assay B	36.3

did the presence of the individual metal ions or a mixture of all of them in the isolation medium result in any greater preservation of the permeability of the chloroplast membrane than the tris-sucrose solution alone, provided the required low temperature (referred to in the Methods section) was scrupulously maintained.

Relationship Between Isolation and Assay Medium and Dark CO_2 Fixation. Recently, Walker (23) using a new isolation method and assay medium reported improved rates of light-dependent CO_2 fixation. Therefore, we compared his method of isolation and assay with that reported in this paper for ATP-dependent CO_2 fixation (table IV). It is clear that the maximum activity for dark CO_2 fixation is observed only with chloroplasts isolated and assayed according to the procedure outlined in this paper. Chloroplasts isolated in the sorbitol mixture and assayed in the mixture described in this paper manifested about 75 % of the activity of those isolated and assayed according to our procedure. It is clear from the data that the composition of the assay medium seemed to be a more critical factor than that of the isolation medium.

It might be argued that the difference in the activity of the 2 preparations resides in the retention of permeability characteristics of the chloroplast membrane. If the sorbitol-isolated chloroplasts were less permeable to ATP and ribose-5-P than the sucrose-tris isolated chloroplasts, the lesser activity on the part of the former could be explained on that basis. However, the same difference in ATP-dependent CO_2 fixing activity prevailed in the chloroplast extract where membrane permeability was not a factor. This suggested a limiting cofactor in Walker's system for dark CO_2 fixation. When each component of the sorbitol assay system at the concentration recommended by Walker (23) was tested separately in an otherwise complete tris system, sodium chloride, isoascorbate, and phosphate had no effect on ATP-dependent CO_2 fixation. Sorbitol and $MnCl_2$ inhibited slightly; and EDTA and cysteine stimulated the tris system (as already shown in the above sections). The concentration of Mg^{2+} used by Walker (0.8 mM) in contrast to the 60 mM in our system profoundly reduced the amount of CO_2 fixation observed. This was almost precisely the amount of fixation observed in the sorbitol-isolated, sorbitol-assayed chloroplast system. It should be mentioned that when the specific activity in table IV is calculated as μmoles of CO_2-fixed/mg chlorophyll $\times$ hour, it is clear that the 24 μmoles of CO_2-fixed/mg chlorophyll $\times$ hour reported by Walker is the most dark fixation one could observe at that Mg^{2+} concentration, regardless of the amount of ATP generated through photophosphorylation. It is possible that the potential efficiency of fixation was higher, but the actual fixation was limited by the Mg^{2+} concentration. Further, Avron's (4) magnesium titration curve for photophosphorylation in extracts of chard clearly shows that the optimum magnesium concentration for photophosphorylation in chard chloroplasts is limiting for the ATP-dependent CO_2 fixing system. The optimum concentration of Mg^{2+} for ATP-dependent CO_2 fixation in chard chloroplasts is inhibitory to photophosphorylation. It would seem that the best approach to obtaining maximum light-dependent CO_2 fixation in chloroplasts from higher plants would be to allow photophosphorylation to proceed first at the optimum magnesium concentration for that process (which fortunately is lower than that required for ATP-dependent CO_2 fixation). After sufficient time is allowed for ATP formation, then the components for CO_2 fixation could be added to the system with the increased amount of magnesium necessary for that process.

Recently, Jensen and Bassham (17) prepared spinach chloroplasts which exhibit rates of light-dependent CO_2 fixation approaching the observed light-dependent levels in the whole leaf (63 % of the in vivo photosynthetic rate). These rates are the highest yet reported. The full potential of the carboxylative phase enzymes present in chloroplasts is even higher as has been shown by the addition of ribose-5-P and ATP. The bicarbonate concentration required for the maximum rate of ATP-dependent CO_2 fixation as reported above is higher than that required for light-dependent CO_2 fixation. Since the concentration of CO_2 or bicarbonate in the intact leaf at the enzyme site is not known, the significance of this difference for the true in vitro systems is not apparent. However, the high rate of ATP-dependent CO_2 fixation reported here can be sustained for 45 minutes to an hour whereas the rate of the light-dependent system drops off rapidly after 10 minutes (17).

Phosphoenolpyruvate Carboxylase Activity in Spinach Fractions. It is important to determine that all the observed incorporation is due to the activity of the ribulose-1, 5-diP carboxylase because it is possible that other mechanisms such as P-enolpyruvate carboxylase which has been reported to be present in extracts of spinach chloroplasts (20) could lead to CO_2 incorporation into acid-stable compounds. Table V shows that the fixation observed in the chloroplast fraction could not be due to P-enolpyruvate carboxy-

Table V. *Relative Activities of Ribulose-1, 5-diphosphate Carboxylase and Phosphoenolpyruvate Carboxylase*

The complete system contained in μmoles: tris-HCl buffer, pH 7.5, 100; ribulose-1, 5-diP, 2 or P-enolpyruvate, 10; $MgCl_2$, 160; and $Na_2{}^{14}CO_3$, 30 (2.5×10^5 cpm/μmole). Total volume was 2.7 ml. Incubation time was 10 min at 30° in an atmosphere of helium.

	Ribulose-1, 5-diphosphate carboxylase	Phosphoenolpyruvate carboxylase
	μmoles CO_2 fixed min^{-1} $mg\ protein^{-1}$	
Washed chloroplasts	13.2	1.8
Chloroplast extract	16.8	0.6
144_g90 (cytoplasm)	12.0	9.6

lase since the activity observed either with whole chloroplasts or with the chloroplast extract was only one-seventh or less than that observed with ribulose-1, 5-diP. There was, however, considerable P-enolpyruvate carboxylase activity in the cytoplasm. Although the P-enolpyruvate carboxylase activity observed was only one-seventh or less than that of the ribulose-1, 5-diP carboxylase activity, the P-enolpyruvate carboxylase activity in the chloroplasts is considerably greater than that reported by Rosenberg et al. (20). If the activity reported by these workers were only four-tenths of the true activity, as they suggest, then their maximum activity still would have been only 0.5 μmole of CO_2 fixed/mg chlorophyll × hour. The activity observed with washed chloroplasts isolated as described in this paper is 20 μmoles of CO_2 fixed/mg chlorophyll × hour, and there was considerably more P-enolpyruvate carboxylase in the cytoplasmic fraction, as would be expected.

Further evidence that the activity observed in the chloroplasts is produced primarily by ribulose-1, 5-diP carboxylase was obtained in the following manner. An extract of chloroplasts was prepared (tris buffer with 0.001 M dithiothreitol) and passed over a column of G25 coarse sephadex equilibrated with the same buffer. This treatment removed any endogenous CO_2 acceptors as well as any reduced pyridine nucleotides except those tightly bound to protein. The material was then eluted from the column with tris buffer with 0.001 M dithiothreitol. This extract had the same specific activity as that observed in the chloroplasts made permeable to ATP and ribose-5-P. Thin-layer chromatography and radioautography of this system revealed 90 % or more of the radioactivity in glyceric acid 3-P.

Conclusions

Washed whole chloroplasts retained their permeability characteristics as determined by the physiological technique of measuring CO_2 fixation in the presence of ATP and ribose-5-P. The absence of CO_2 fixation in the presence of the phosphorylated compounds was interpreted as a failure of at least 1 of these compounds to penetrate the chloroplast since extracts of these same chloroplasts fixed large quantities of CO_2 in the presence of ribose-5-P and ATP. The integrity of the chloroplast membrane, as measured by the fixation of CO_2 in the presence of ribose-5-P and ATP in the whole chloroplast as compared with the chloroplast extract, was preserved in a tris-HCl buffer containing 0.5 M sucrose. An effect similar to, but less pronounced than, that produced by the presence of sucrose in the isolation medium was caused by the presence of $MnCl_2$ or $CaCl_2$ in the buffered sucrose isolation medium. The addition of KCl enhanced slightly the effect produced by the addition of sucrose alone to the isolation medium. The addition of $MgCl_2$ to the isolation medium caused either a leakage of enzymes out of the chloroplast into the cytoplasm, or the chloroplast to become more fragile and rupture with release of enzymes into the cytoplasm.

The addition of EDTA to the chloroplast suspension assayed in tris without sucrose resulted in increased permeability and/or preservation or stimulation of activity, as did the addition of dithiothreitol. It is not possible at this time to distinguish between the separate effects. It is clear, however, that the effect of EDTA and dithiothreitol in preserving or stimulating the activities are quantitatively equal, but not additive.

There was detectable P-enolpyruvate carboxylase activity in the chloroplasts, but at only about one-seventh the level of the ribulose diphosphate carboxylase activity. There was 5 times as much P-enolpyruvate carboxylase activity in the cytoplasm as in the chloroplast.

The specific activity for the carboxylative phase of ATP-dependent CO_2 fixation in these chloroplasts can be as high as 329.6 μmoles of CO_2/mg chlorophyll × hour and, therefore, ribulose-1, 5-diP carboxylase cannot be limiting.

Acknowledgments

The fine technical assistance of Joanne Stevenson, Nellie Whelan, and Henry Mack is gratefully acknowledged.

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