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Measurement of Ribulose 1,5-Bisphosphate from Spinach Chloroplasts[1]

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ABSTRACT

A technique has been developed for the rapid and simple measurement of ribulose 1,5-bisphosphate from isolated spinach chloroplasts. The endogenous ribulose bisphosphate was detected enzymically using $^{14}CO_2$ and ribulose bisphosphate carboxylase/oxygenase released from the chloroplasts. Ribulose 5-phosphate kinase was inhibited with 0.4 to 0.6 millimolar 2,6-dichlorophenol-indophenol and 4 micromolar carbonyl cyanide *m*-chlorophenylhydrazone. Phosphoenolpyruvate carboxylase activity was low with washed chloroplasts and its labeled product, [^{14}C]oxalacetate, was destroyed by heating with 1.0 N HCl at 90 C. The assay method was linear from 0.05 to 0.87 nanomoles ribulose bisphosphate per milliliter. The latter value was determined with chloroplast material having 44 micrograms of chlorophyll per milliliter. This technique was simple and direct, used less chloroplast material, yet provided results comparable to a previously described enzymic technique in which ribulose bisphosphate was determined after the precipitation of chloroplast proteins by perchloric acid.

In previous studies, RuBP[3] levels in leaf material, algae, and isolated chloroplasts have been determined by two methods. The first method, developed for determination of the intermediates of the photosynthetic carbon reduction pathway, consisted of labeling the products of photosynthesis with ^{14}C or ^{32}P. Separation of products was achieved by two-dimensional paper chromatography (14, 23) or, more recently, by ion exchange liquid chromatography (10, 15). With isolated chloroplasts, however, steady-state carbon flow during photosynthesis is not easily attained, making it difficult to determine the specific activities and total amounts of pathway intermediates.

The second procedure was based upon the precipitation of chloroplast proteins by $HClO_4$, followed by neutralization and enzymic analysis. RuBP was detected by $^{14}CO_2$ uptake upon the addition of RuBP carboxylase. Although this method has the sensitivity of an isotopic technique, it often requires more chloroplast material than is convenient, and also requires extensive manipulations of the samples (acidification, neutralization, centrifugation) prior to assay. Alternately, RuBP detection has been coupled with an enzymic assay to NADH oxidation (17). This technique converts PGA by way of dihydroxyacetone phosphate to α-glycerophosphate so that four NADH are produced per RuBP molecule consumed. The sensitivity of this technique is limited by the measurement of small optical density changes.

We describe a method for the direct measurement of endogenous chloroplast RuBP which uses *in situ* RuBP carboxylase from lysed chloroplasts. Inhibition of Ru5P kinase is achieved in a mild oxidizing environment. This method rapidly and conveniently measures chloroplast RuBP to levels below 0.1 nmol per sample.

MATERIALS AND METHODS

Preparation of Chloroplasts. Chloroplasts were isolated from 6-week-old spinach plants (*Spinacia oleracea* var. *Viroflay*) as described previously (1). In these preparations 70 to 95% of the chloroplasts were intact as measured by ferricyanide-dependent O_2 evolution (3, 11). Chl was determined according to Vernon (22). RuBP was synthesized from Ri5P using a chloroplast extract maintained under N_2 without CO_2 or O_2, and with an ATP-generating system (1).

Measurement of Chloroplast RuBP. Chloroplast RuBP was determined by measuring the total RuBP and subtracting the RuBP external to the chloroplasts. In 1.5-ml capped polypropylene centrifuge tubes, 0.1 ml of chloroplasts (usually more than 10 μg Chl) was incubated with 0.9 ml of 25 mM Hepes-NaOH (pH 8.0), 20 mM $MgCl_2$, 0.4 to 0.6 mM DCPIP, 4.0 μM CCCP, and 10 mM $NaHCO_3$ of 5.0 μCi/μmol. The reactions were terminated after 10 to 30 min with 0.4 ml of 1.0 N HCl. The colored particulate matter was removed by centrifugation in a Brinkmann microcentrifuge for 5 min and 1.0 ml of the supernatant was transferred to a scintillation vial, dried at 90 C to remove unreacted $^{14}CO_2$, and counted. The CCCP stock solution (0.1 mM) was prepared in ethanol. The concentration of DCPIP was determined at 610 nm upon reduction with dithionite, where μmol DCPIP/ml = 0.0563 (ΔA) (16). The DCPIP concentration was 0.6 mM when RuBP was measured from illuminated or actively photosynthesizing chloroplasts.

Assays performed with less than 10 μg Chl/ml were supplemented with 0.1 to 0.2 mg of purified tobacco RuBP carboxylase or with a chloroplast extract containing RuBP carboxylase. Crystalline tobacco enzyme was prepared according to Lowe (12). Prior to use, the crystals were collected by centrifugation and dissolved in 25 mM Bicine-NaOH (pH 8.6), 20 mM $MgCl_2$, 10 mM NaCl, and 10 mM $NaHCO_3$. These preparations were heat-activated for 20 min at 50 C according to Singh and Wildman (20). The extract was prepared by lysing chloroplasts (0.3–0.6 mg Chl) in 1.0 ml of 25 mM Hepes-NaOH (pH 8.0) and 20 mM $MgCl_2$. After centrifugation the supernatant was left at room temperature for 30 min to remove all RuBP. This crude extract typically contained 0.1 to 0.2 mg of protein and undetectable amounts of RuBP (<0.002 nmol) in 0.05 ml.

External RuBP was determined by centrifuging 0.15 ml of a chloroplast suspension in a Beckman microfuge for 15 s and assaying 0.1 ml of the supernatant for RuBP with DCPIP and

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[3] Abbreviations: RuBP: ribulose 1,5-bisphosphate; PGA: 3-phosphoglycerate; Ru5P: ribulose 5-phosphate; Ri5P: ribose 5-phosphate; DCPIP: 2,6-dichlorophenol-indophenol; CCCP: carbonyl cyanide *m*-chlorophenylhydrazone; PEP: phosphoenolpyruvate; OAA: oxalacetate.

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CCCP as above using either purified tobacco or crude chloroplast RuBP carboxylase. When possible, sample sizes were used having more than 0.1 nmol RuBP/ml.

For comparison, chloroplast RuBP was also determined by perchloric acid treatment according to Latzko and Gibbs (7, 8). The chloroplast suspension, containing about 0.1 mg Chl, was acidified with an equal volume of 10% $HClO_4$ and the sample was cooled immediately to 4 C and centrifuged at 2,000*g* for 5 min. The supernatant was made 0.1 M with Bicine and sufficient 4 N KOH was added to yield a pH of 7.8 to 8.0. RuBP was stable at this pH for several hours at ice temperatures. $KClO_4$ was removed by centrifugation and RuBP was assayed for 1 h at 25 C by adding 0.5 ml of the neutralized extract to 0.2 ml of purified tobacco RuBP carboxylase. The assay contained 7.1 mM $MgCl_2$, 9.3 mM $NAH^{14}CO_3$ (5.0 μCi/μmol), 0.1 to 0.15 mg of tobacco RuBP carboxylase, and about 60 mM Bicine-KOH (pH 7.8 to 8.0) as provided by the neutralized extract. The reactions were terminated with 0.5 ml of 1 N HCl and the samples were dried and counted by liquid scintillation. RuBP external to the chloroplast was also measured by the $HClO_4$ method after removal of the plastids by centrifugation.

RESULTS

Inhibition of Ru5P Kinase by DCPIP and CCCP. Chloroplast Ru5P kinase is activated in response to light or by thiol compounds such as DTT (5, 6). Furthermore, the activity of this enzyme is inhibited by reagents that react with sulfydryl groups (iodoacetamide and O_2) (6). We find that DCPIP, acting as a mild oxidant in air, effectively inhibits spinach chloroplast Ru5P kinase upon lysis (Tables I and II).

In the dark, 1.46 nmol CO_2 were fixed when chloroplasts having 25 μg Chl were lysed in pH 8.0 buffer containing Mg^{2+} and $^{14}CO_2$ (Table I). This fixation of CO_2 was catalyzed by RuBP carboxylase released from the chloroplasts using endogenous RuBP, plus RuBP formed from other intermediates by the action of Ru5P kinase. The addition of CCCP (4.0 μM) and DCPIP (0.4 mM) reduced the amount of CO_2 fixed to 0.94 nmol. With 1.0 nmol each of Ru5P and ATP added to the assay, 0.73 nmol was recovered in the control (2.19 minus 1.46 nmol). However, there was essentially no increase in counts if DCPIP and CCCP were both present in the assay (0.96 *versus* 0.94 nmol). Under these conditions, Ru5P kinase was effectively inhibited by the combined action of CCCP and DCPIP. Added RuBP (1.40 nmol) was quantitatively recovered as the ^{14}C-labeled product (2.34 minus 0.94 nmol), indicating that CCCP and DCPIP did not interfere with the assay of this amount of RuBP.

The assay of chloroplast RuBP with 0.4 mM DCPIP and 4.0 μM CCCP was about 90% completed within 10 min (Fig. 1). Over half of the chloroplast RuBP was consumed in the 1st min. Similar results were obtained when more RuBP (1.4 nmol) was added to the assay (data not shown). The presence of purified tobacco RuBP carboxylase may have increased the initial rate of RuBP carboxylation, but did not affect the amount detected after 5 min.

Table I. *Effect of CCCP and DCPIP on Measurement of RuBP from Lysed Chloroplasts*

RuBP was assayed by $^{14}CO_2$ fixation with 25 μg Chl in 0.5 ml for 20 min. The values in parentheses represent nmol RuBP/mg Chl.

Experimental Conditions	No CCCP or DCPIP	4.0 μM CCCP	
		mM DCPIP 0.0	0.4
	nmol CO_2 fixed		
No additions	1.46 (58.4)	1.06 (42.4)	0.94 (37.6)
1.0 nmol Ru5P and 1.0 nmol ATP	2.19	1.33	0.96
1.0 nmol Ru5P, 1.0 nmol ATP and 1.4 nmol RuBP	3.71	2.76	2.34
1.4 nmol RuBP	2.94	2.50	2.35

Table II. *Effect of Various Metabolites on the Detection of Chloroplast RuBP*

Assays were conducted in 0.5 ml with 4.0 μM CCCP and 18.4 μg Chl for 20 min.

Intermediates		mM DCPIP 0	0.4
	nmol	*nmol CO_2 fixed*	
None	—	0.34	0.21
RuBP	1.25	1.58	1.43
Ru5P	1.85	0.54	0.22
Ri5P	3.00	0.42	0.21
Ru5P, ATP	3.00, 3.00	1.03	0.23
Ri5P, ATP	3.00, 3.00	0.70	0.22
ATP	3.00	0.39	0.22
FBP[a]	1.50	0.36	0.23
PGA	3.00	0.35	0.24
PEP	5.00	0.57	0.29
Pyr[b]	1.50	0.47	0.25

[a] FBP: fructose 1,6-bisphosphate.

[b] Pyr: pyruvate.

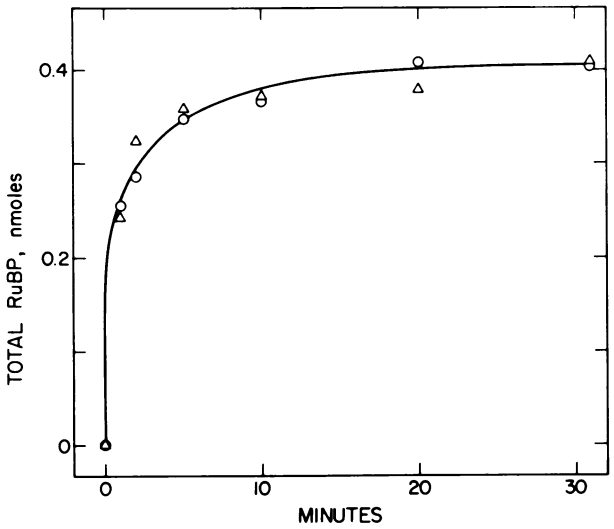

FIG. 1. Time course of RuBP assay. Chloroplasts (22 μg Chl) in 0.05 ml were incubated with 0.45 ml of assay medium containing 4.0 μM CCCP and 0.42 mM DCPIP, either with (△) or without (○) added tobacco RuBP carboxylase (142 μg). At the times indicated 0.05 ml samples were added to 0.5 ml of 1 N HCl to stop the reaction.

PEP Carboxylase Activity in Spinach Chloroplast Preparations. The predominant carboxylase in isolated spinach chloroplasts was RuBP carboxylase, however, some PEP carboxylase, usually considered to be a cytoplasmic enzyme, was also present. Because of this activity, the addition of several chloroplast intermediates resulted in an apparent small increase in CO_2 fixation (Table II). Note that in the presence of 5 nmol PEP, only 0.08 nmol more CO_2 was fixed, indicating that PEP carboxylase activity was low. If the OAA formed by PEP carboxylase had been trapped as aspartate or malate, more counts would have resulted (13). The samples were treated with 1.0 N HCl and dried at 90 C before counting to remove unfixed CO_2. This caused most of the labeled OAA to be decarboxylated.

Limits of RuBP Detection. The data shown in Tables I and II indicated that DCPIP did not affect the measurement of total RuBP. However, a marked inhibition of the initial reaction kinetics of carboxylation was observed (Table III). Increasing concentrations of DCPIP reduced the level of RuBP carboxylase activity detected 60 s after chloroplast lysis in Hepes buffer at pH 8.0. The assay contained saturating levels of CO_2, Mg^{2+} and RuBP.

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Table III. *Effect of DCPIP and CCCP on Activity of RuBP Carboxylase*

The rate of CO_2 fixation by chloroplast RuBP carboxylase was measured upon lysis of chloroplasts at 25 C (1). Chloroplasts (14.6 μg Chl) were lysed in 0.5 ml of 25 mM Hepes-NaOH (pH 8.0), 20 mM $MgCl_2$, 0.3 mM RuBP, and 10 mM $NaHCO_3$, 0.5 μCi/μmol with CCCP and DCPIP as indicated. Reaction was stopped after 30 s.

No CCCP or DCPIP	4.0 μM CCCP				
	mM DCPIP				
	0.0	0.4	0.8	1.2	1.6
	nmol CO_2 fixed/mg Chl·min				
3.85	3.27	1.89	1.43	0.98	0.70

Table IV. *Relationship of DCPIP and Amounts of Chloroplasts on Analysis of RuBP*

Assays were conducted in 0.5 ml with 4.0 μM CCCP as described under "Materials and Methods" for 20 min. The values in parentheses represent nmol of RuBP/mg Chl.

Experiment	Chloroplasts	mM DCPIP			
		0	0.4	0.8	1.6
	μg Chl	*nmol CO_2 fixed*			
A.	5.8	0.14 (24)	0.12 (21)	0.10 (17)	0.09 (16)
	14.5	0.30 (21)	0.28 (19)	0.29 (20)	0.27 (19)
	29.0	0.68 (23)	0.58 (20)	0.59 (20)	0.61 (21)
B. +11.4 nmol RuBP	5.8	11.60	11.22	8.24	5.03
	14.5	11.77	11.68	11.50	10.75
	29.0	12.34	11.97	11.94	11.50

Because the activity of the RuBP carboxylase was reduced by DCPIP, chloroplast RuBP was underestimated in assays performed with small amounts of chloroplast material (5.8 μg Chl) and 0.8 or 1.6 mM DCPIP (Table IVA) or high RuBP (Table IVB). Addition of more chloroplasts overcame the problem, suggesting that it was caused by insufficient RuBP carboxylase activity. Complete measurement of RuBP was also obtained by supplementing assays having less than 10 μg Chl/ml with purified tobacco enzyme (data not shown). Because RuBP carboxylase was slowly inactivated by DCPIP, the purified enzyme was added to the assay after the chloroplast sample. Assays having chloroplasts with more than 10 μg Chl/ml usually have sufficient carboxylase activity for complete RuBP detection.

The RuBP assay was linear over a range of 2.2 to 44.2 μg Chl/ml, which corresponded to 0.05 to 0.87 nmol RuBP/ml (Fig. 2). With 0.05 nmol RuBP, 550 dpm were obtained when assayed with $NaH^{14}CO_3$ having a specific activity of 5.0 μCi/μmol suggesting that the technique as outlined under "Materials and Methods" was limited by insufficient counts for liquid scintillation detection.

Comparison with $HClO_4$ Method. The RuBP assay technique reported here compared quite favorably with that used by Latzko and Gibbs (7, 8) where protein was first removed by treatment with $HClO_4$. Because of the dilution steps involved, the $HClO_4$ procedure required at least five times more chloroplast material than the method described here. With photosynthesizing chloroplasts, the technique for measuring RuBP with DCPIP and CCCP gave results comparable to those obtained with the $HClO_4$ procedure. Figure 3 shows that as RuBP levels in the chloroplast changed from 8 to 39 nmol/mg Chl the two methods were within 10% of each other, except after 3 min of illumination. This difference at 3 min may have been real because the $HClO_4$ sample was taken 2 to 4 s after the initiation of the DCPIP assay. In isolated chloroplasts, rapid fluctuations in the RuBP pool occur during the first 4 min of photosynthesis (19).

DISCUSSION

Because the pool size of RuBP is small when compared to the rate of turnover during photosynthesis, only a few seconds would be required for large changes in RuBP levels to occur. Consequently, it is essential to stop the metabolic processes in chloroplast preparations rapidly in order to inhibit changes in RuBP prior to assay. The most sensitive of the previous enzymic methods for measuring RuBP in plant material relied on $HClO_4$ for this purpose (7, 8). The present method measures RuBP upon chloroplast lysis, using chloroplast RuBP carboxylase and the dye, DCPIP in the oxidized form, to inhibit Ru5P kinase activity. It seems that chloroplast lysis upon a 10-fold dilution into low osmotic medium is sufficiently rapid to capture the RuBP pool accurately.

The data in Tables I and II and in Figure 3 indicate that CCCP and DCPIP rapidly and efficiently eliminated Ru5P kinase activity. In the chloroplast the pentose-monoP pool is usually less than 20% of the RuBP pool (14) suggesting that its contribution to the synthesis of RuBP would be small before complete inhibition of the Ru5P kinase occurred. Experimentally, this was observed where the addition of various intermediates of the photosynthetic carbon reduction cycle, including pentose phosphates, added little to the amount of RuBP which was measured (Table II).

RuBP was detected as a linear function of the amount of chloroplast material (Fig. 2). The lowest amount of RuBP detected was 0.05 nmol, but more reproducible results were obtained with samples containing at least 0.1 nmol RuBP/ml. Although longer assay times for chloroplast RuBP measurements were used in this report, the reaction was 90% completed within 10 min (Fig. 1).

The addition of PEP to the RuBP assay resulted in an increase in counts, suggesting the presence of PEP carboxylase activity (Table II). This was not a serious problem with our assays for several reasons. First, in C_3 plants, PEP is present in small amounts and PEP carboxylase activity is low (2, 9). Second, spinach PEP carboxylase is of the high K_m, low V_{max} type (13, 18, 21). Third, PEP carboxylase is not an enzyme of the chloroplast and is a minor contaminant of washed spinach chloroplast preparations. Fourth, the labeled OAA formed is acid- and heat-labile, breaking

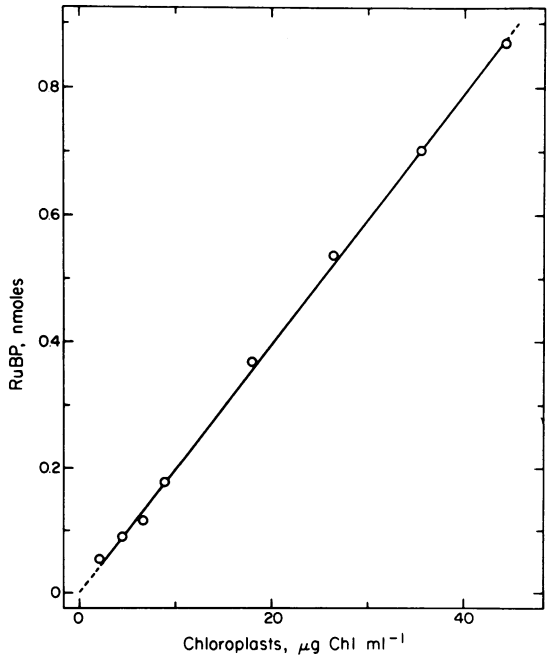

FIG. 2. Relation between amounts of spinach chloroplast material and the amount of RuBP. Chloroplasts were lysed in 1.0 ml of assay medium and stopped at 20 min. The amount of RuBP is the difference between the total RuBP and the external RuBP. External RuBP (1.36 nmol/mg Chl) was determined with purified tobacco RuBP carboxylase and 0.05 ml of suspending medium following centrifugation and removal of the chloroplasts.

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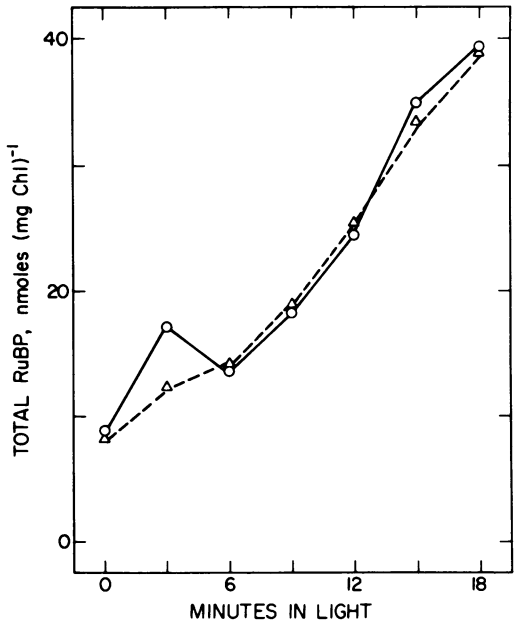

FIG. 3. RuBP from chloroplasts in the light as determined by the $HClO_4$ technique and by methods of this paper. Chloroplasts (88 μg Chl) were illuminated (800 $\mu E/m^2 \cdot s$) in 0.7 ml of solution C (4) with 7.1 mM $NaHCO_3$ and 1.4 mM PPi. When indicated, samples having 0.1 ml were dispensed into 0.9 ml of assay medium containing 4.0 μM CCCP and 0.6 mM DCPIP, and supplemented with 0.05 ml of spinach chloroplast RuBP carboxylase (○). Immediately following removal of the above sample, the remaining chloroplast suspension (0.6 ml) was acidified with an equal volume of 10% $HClO_4$ and assayed for RuBP (△).

down almost entirely during the 90 C heating step with 1.0 N HCl. This assay technique must be used with caution when assaying plant material, such as chloroplasts from C_4 species, in which PEP carboxylase activity might contribute to measurements of CO_2 fixation into acid-stable products.

Some precautions must be taken with this technique. The activity of RuBP carboxylase is inhibited by high concentrations of DCPIP (Table IV), therefore, it may be necessary to add additional enzyme to the assay. Provided that critical enzyme concentrations are maintained and efforts are taken to reduce counts contributed by PEP carboxylase to the assay, RuBP levels can be determined directly upon lysis of spinach chloroplasts in the presence of DCPIP and CCCP. The simplicity and accuracy of this technique, coupled with the convenience of small sample size, overcome its possible shortcomings.

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