Since, however, cut D in Fig. 1 contains one of the stronger bands, larger amounts of plasma would need to be applied to the gel for accurate estimations in this way of the weaker bands. Fortunately, however, certain estimations such as immunological tests in gels[6] or scintillation counting for radioactivity are unaffected both by the considerable quantities of soluble starch or the much lower amounts of protein derived from the gel which are present in the eluates made as described above. If necessary soluble starch can be eliminated from the eluate by chromatography on Dowex-2 by the method of DE PAILLERETS *et al.*[7].

ACKNOWLEDGEMENTS

I am grateful to Dr. S. COHEN who carried out some of the initial experiments and to Mr. L. ASSEMAKIS for criticism and skilled technical assistance.

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D-THREOSE 2,4-DIPHOSPHATE INHIBITION OF 3-PHOSPHOGLYCERIC ACID PHOTOREDUCTION BY A SONICALLY RUPTURED SPINACH CHLOROPLAST SYSTEM

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(Received December 15, 1959)

SUMMARY

1. [1-^{14}C]PGA is readily reduced by a sonically ruptured chloroplast system in light. This reduction is inhibited by threose 2,4-diphosphate.

2. PGA accumulates when $^{14}CO_2$ is incubated in light with the sonically ruptured chloroplast system in the presence of threose 2, 4-diphosphate. This indicates that PGA is in the major pathway of photosynthetic CO_2 fixation in the system.

3. Threose diphosphate inhibition of the total $^{14}CO_2$ fixation by the carbon cycle of sonically ruptured spinach chloroplasts is not due to inhibition of carboxydismutase, but due primarily to inhibition of triose phosphate dehydrogenase which in turn limits the formation rate of the photosynthetic carbon cycle CO_2 acceptor, RuDP.

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INTRODUCTION

The first stable intermediate formed by $^{14}CO_2$ fixation in illuminated photosynthetic systems is PGA[1]. Recently, an alternative path has been suggested for photosynthetic CO_2 fixation which does not involve PGA.[2] In this suggested path, carboxylation of RuDP yields a six carbon branched chain acid which subsequently rearranges without cleavage to PGA, to form a six carbon chain directly. If it were possible to block the reduction of PGA to triose in the photosynthetic carbon cycle, then it should be possible to observe whether CO_2 still flows into the cycle via some pathway other than that involving PGA.

Recently threose 2,4-diphosphate was shown to inhibit specifically muscle and yeast triose phosphate dehydrogenase having DPN as a cofactor[3,4]. This inhibitor has a K_i of $2 \cdot 10^{-7}$ for the oxidation reaction. The reduction reaction is less sensitive to the inhibitor, a concentration of about $2 \cdot 10^{-5}$ M yielding 50% inhibition. The inhibition of the enzyme is apparently due to the similarity of the threose diphosphate and 1,3-diphosphoglyceric acid molecules. It was of interest to determine whether threose diphosphate would also inhibit triose phosphate dehydrogenase in a TPN requiring photosynthetic system. Such an inhibition would then make it possible for PGA to accumulate when $^{14}CO_2$ was photosynthetically fixed in the presence of threose diphosphate. Since the photosynthetic carbon cycle involves a number of enzymic steps other than triose phosphate dehydrogenase, it was of interest to find whether some of these other enzymes were also inhibited by threose diphosphate. The experiments reported in this paper were done with a spinach chloroplast system since phosphorylated intermediates such as PGA and threose diphosphate do not readily penetrate intact cells. Whole chloroplasts were also found to be somewhat impermeable to the phosphorylated substrates and cofactors used in these experiments, confirming the observations of TOLBERT[5]. We found that sonic rupture of whole chloroplasts removed the permeability barrier and increased the rate of CO_2 fixation on a unit chlorophyll basis. Sonically ruptured chloroplasts proved more suitable for these experiments than either whole or broken chloroplasts as prepared by ARNON[6].

PROCEDURES

Whole chloroplasts were prepared from the leaves of *Spinacea oleracea* by grinding in 0,5 M sucrose buffer and were purified by fractional centrifugation according to the method described by HOLM-HANSEN *et al.*[7]. The whole, washed chloroplasts were resuspended in about 20 times their own volume of 10^{-3} M potassium phosphate buffer, pH 7.4. The chloroplast suspension was then sonically ruptured at 0° for 90 sec in a 9 kc, 100 W Raytheon magnetostriction oscillator operated at full voltage. The sonically ruptured suspension was used directly in the experiments. Investigation of this material with the light microscope showed no intact chloroplasts, and only a few large chloroplast fragments. Chlorophyll concentrations in the sonically ruptured suspension were determined spectrophotometrically in 80% acetone according to ARNON[8].

Abbreviations used: ADP, adenosine diphosphate; GSH, glutathione; HOAc, acetic acid; PEP, phosphoenolpyruvate; PGA, 3-phosphoglyceric acid; RuDP, ribulose 1,5-diphosphate; SAS, saturated ammonium sulfate; TPN, triphosphopyridine nucleotide; TPP, thiamine pyrophosphate; Tris, tris(hydroxymethyl)aminomethane; DPN, diphosphopyridine nucleotide.

The organic cofactors and metal cofactors were prepared separately and titrated to pH 7,4. The photosynthetic reactions were run at 24° and 1000 foot candles in airtight 35-ml flasks. The total reaction mixture volume was about 1.5 ml and contained about 1 mg of chlorophyll.

After 30-min incubation the enzymic reaction was stopped by addition of 4 ml of ethanol to the reaction mixture. The precipitate was then extracted with 20% ethanol, 100% ethanol and water successively. The extracts were pooled giving a total volume of ~ 10 ml. A small aliquot was acidified and placed on an aluminum planchet for assay of the total radioactivity in the reaction mixture. Another aliquot was applied to the origin of a paper chromatogram.

Two dimensional chromatography of the photosynthetic products and counting of the radioactive areas on the chromatograms was carried out according to the methods described by BASSHAM and CALVIN[1].

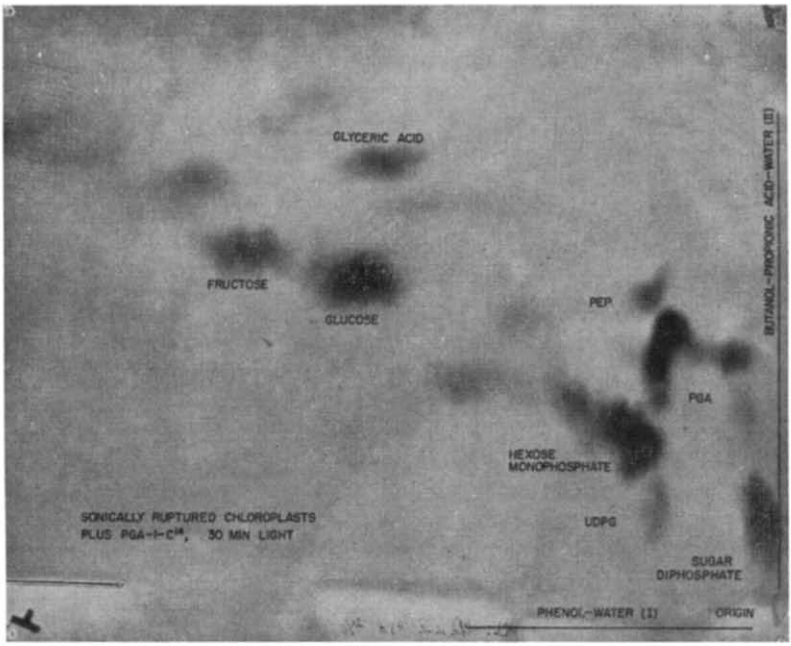
Carboxydismutase was prepared from *Tetragonia expansa* chloroplasts. These were prepared in 0.5 *M* sucrose and suspended in dilute buffer according to the method of SOGO *et al.*[9]. Carboxydismutase was obtained by ammonium sulfate fractionation of the clear supernatant (termed "chloroplast extract" in ref. 9) obtained from centrifugation of the chloroplast suspension. The procedure of MAYAUDON[10], normally used for the partitial purification of the crude extract of *Tetragonia expansa*, was modified for fractionation of the chloroplast extract, the material precipitating between 0.34 to 0.49 SAS being chosen for use in these experiments.

Carboxyl-labeled PGA was prepared by incubating RuDP with $NaH^{14}CO_3$ in the presence of carboxydismutase[11] and Mg^{++} (see ref. 12, 13). The reaction mixture consisted of 12.7 μM $NaH^{14}CO_3$ (300μC), 120 μM $MgCl_2$, 900 μM Tris at pH 8.3, approx. 3 μM RuDP, and 1.4 mg of enzyme preparation. The final volume of the incubation mixture was 2.25 ml. Incubation was carried out at room temperature for 20 h. The yield of radioactive PGA was about 1.5 μM, or 50 % of the theoretical value. The [1-^{14}C]PGA was purified by chromatography on washed No. 4 Whatman paper using a butanol–propionic acid–water system. The [^{14}C]PGA was located by radioautography and was recovered by elution. The eluate was taken to dryness and redissolved in 0.5 ml of 0.0402 *N* $NaH^{12}CO_3$.

D-threose 2,4-diphosphate was generously provided by Dr. BALLOU and Dr. FLUHARTY of the University of California Biochemistry Department.

EXPERIMENTAL RESULTS AND DISCUSSION

Preliminary experiments were done in which [1-^{14}C]PGA was incubated with the sonically ruptured chloroplast system in light and dark with the cofactors given in Table I. Chromatograms from this experiment (Fig. 1) showed that reduction of [1-^{14}C]PGA to triose phosphate in this enzyme system is a light dependent process. In the dark [1-^{14}C]PGA is not reduced. This accounts for the relatively large amounts of glyceric acid and alanine in the dark experiment. When [1-^{14}C]PGA reduction was established as a light dependent process in the system, threose diphosphate inhibition of spinach chloroplast triose phosphate dehydrogenase was investigated by incubating [1-^{14}C]PGA with sonically ruptured spinach chloroplasts in light in the presence and absence of threose 2,4-diphosphate. The reaction mixtures for this experiment are given in Table I. After two dimensional paper chromatography of the reaction mixture extract



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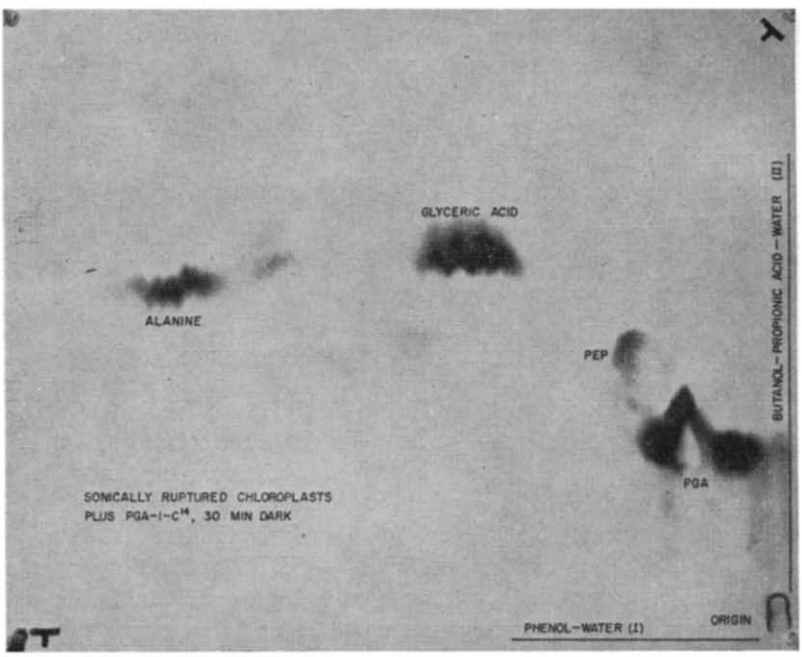

b

Fig. 1. Chromatograms of extracts from sonically ruptured spinach chloroplast systems incubated with [1-^{14}C]PGA in light and dark.

TABLE I

DISTRIBUTION OF ^{14}C AMONG PGA, THE MONOPHOSPHATE SUGARS AND DIPHOSPHATE SUGARS WHEN [1-^{14}C]PGA IS INCUBATED WITH AND WITHOUT THREOSE DIPHOSPHATE IN AN ILLUMINATED CHLOROPLAST SYSTEM

Each reaction mixture contained (amount in μM) $MgCl_2$ 4.0, $MnSO_4$ 1.6, ADP 1.0, TPN 0.02, TPP 1.4, ascorbate 2.5, GSH 1.3, $NaH^{12}CO_3$ 4.0, fragmented chloroplasts 1 ml, [1-^{14}C]PGA 1,200,000 counts/min or approx. 0.1 μM. Threose 2,4-diphosphate 0.12 (present only in one reaction mixture). Total volume 1.275 ml.

	Counts/min on chromatogram	
	— Threose diphosphate	*+ Threose diphosphate*
Total	27,000	23,000
PGA	5,800	12,100
Monophosphates	7,300	1,900
Diphosphates	3,100	800

and location of the ^{14}C containing spots by autoradiography, the activity of the PGA area was compared with the activity of mono- and diphosphate sugar areas. The results of this experiment are given in Table I. The presence of 10^{-4} M threose diphosphate increased the ratio of PGA activity to the activity of the mono- and diphosphate sugar areas which would be expected if triose phosphate dehydrogenase were inhibited during operation of the photosynthetic cycle.

A second experiment was done in which $^{14}CO_2$ fixation was studied in the presence and absence of threose diphosphate. The reaction mixtures were identical to those given in Table I except that 4 μM $NaH^{14}CO_3$ (100 μC) were substituted for the [1-^{14}C] PGA. The results of this experiment ware given in Table II. Again there is an increase in the ratio of PGA activity to phosphorylated sugar activity in the threose diphosphate inhibited system.

The similarity between chromatograms of extracts from $H^{14}CO_3^-$ and [1-^{14}C]PGA incubated systems indicates that PGA is readily assimilated into the $^{14}CO_2$ pathway

TABLE II

DISTRIBUTION OF ^{14}C AMONG PGA, THE MONOPHOSPHATE SUGARS AND DIPHOSPHATE SUGARS WHEN $H^{14}CO_3^-$ IS INCUBATED WITH AND WITHOUT THREOSE 2,4-DIPHOSPHATE IN AN ILLUMINATED CHLOROPLAST SYSTEM

Each reaction mixture contained (amount in μM) $MgCl_2$ 4.0, $MnSO_4$ 1.6, ADP 1.0, TPN 0.02, TPP 1.4, ascorbate 2.5, GSH 1.3, $NaH^{14}CO_3$ 4.0(100 μC), sonically ruptured chloroplasts 1 ml.

	Counts/min on chromatogram per aliquot	
	— Threose diphosphate	*+ Threose diphosphate*
Total	230,000	98,000
PGA	93,000	60,000
Monophosphates	53,000	8,200
Diphosphates	23,000	7,000
	Total counts fixed in the reaction mixture	
Light	16,000,000	5,800,000
Dark	130,000	110,000

of the photosynthetic carbon cycle. The qualitative and quantitative similarity in the effect of threose diphosphate inhibition of the two systems further indicates that PGA is a real and major intermediate in the photosynthetic carbon cycle. While these experiments show that $^{14}CO_2$ photosynthetically fixed by a chloroplast system flows through PGA, they do not settle the question of whether the other three carbon fragment resulting from cleavage of the RuDP carboxylation product is also PGA[14].

An interesting feature of Table II is that the light fixation of $^{14}CO_2$ in the presence of threose diphosphate is about one-third of the control fixation. Inhibition of any of the enzymic steps of the photosynthetic carbon cycle to a rate limiting level would be expected to reduce the total $^{14}CO_2$ fixation by the cycle. The location of threose diphosphate inhibition in the photosynthetic carbon cycle of spinach chloroplast fragments is shown in Table I to be at least in part at triose phosphate dehydrogenase. There is the possibility that 10^{-4} *M* threose diphosphate may also be affecting the rates of other photosynthetic carbon cycle enzymes. Inhibition of most of these enzymes would limit the rate of formation of the acceptor molecule for CO_2 fixation, RuDP. Inhibition of carboxydismutase, however, could also involve direct inhibition of $^{14}CO_2$ entry into the cycle rather than just limitation of ribulose diphosphate formation.

It was of interest for this reason to test the effect of threose diphosphate on carboxydismutase activity both in the crude spinach preparation and in a purified enzyme system isolated from *Tetragonia expansa*. The reaction mixtures and results for these experiments are given in Fig. 2 and Table III. These data show that 10^{-4} *M* threose diphosphate has no effect on the carboxydismutase of the crude system, and inhibits the purified system, by only 15 %.

This result indicates that 10^{-4} *M* threose diphosphate inhibition of $^{14}CO_2$ fixation by this system is not due to inhibition of carboxydismutase which would prevent entry of CO_2 into the cycle, but is primarily due to inhibition of triose phosphate dehydrogenase. The possibility of non-rate-limiting inhibition of some other enzymes acting between triose phosphate and ribulose diphosphate is not, however, ecxluded.

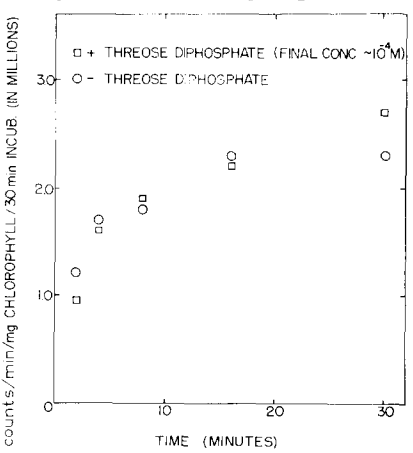

Fig. 2. The time course of $^{14}CO_2$ fixation by carboxydismutase in a sonically ruptured chloroplast system with and without threose diphosphate. Each reaction mixture contained 4.0 μM $NaH^{14}CO_3$ (100 μC), 0.2 μM RuDP and 1 ml of sonically ruptured chloroplasts in 10^{-3} *M* potassium phosphate (pH 7.4) containing 0.59 mg of chlorophyll. Mg^{++}, Mn^{++}, ADP, TPN, TPP, ascorbate and GSH concentrations are given in Table I. The reaction mixtures minus $NaH^{14}CO_3$ were preincubated for 1 min at room temperature after which the bicarbonate was added. The reaction mixtures were shaken in the dark for the prescribed time at 24° in air tight 35-ml vessels. $^{14}CO_2$ fixation by the same enzyme system minus RuDP in light was $21 \cdot 10^6$ counts/min/mg chlorophyll/30 min incubation without threose diphosphate and $7.4 \cdot 10^6$ counts/min/mg chlorophyll/30 min incubation with 10^{-4} *M* threose diphosphate.

The data plotted in Fig. 2 also show that the carboxydismutase rate observed during the first 2 min of the assay is sufficient to account for the rate of $^{14}CO_2$ fixation observed in the light sample.

TABLE III

THE INHIBITION OF PURIFIED CARBOXYDISMUTASE BY THREOSE DIPHOSPHATE

To each reaction mixture were added, in the order listed, the following: water or threose diphosphate solution to make 0.20 ml final volume; $NaH^{14}CO_3$, 1.35 μM; $MgCl_2$, 2 μM; Tris at pH 8.3, 15 μM; carboxydismutase, 23 μg; and RuDP, approx. 0.07 μM. The enzyme was preincubated with the inhibitor, bicarbonate and Mg^{++} at 25° for 2 min. At the end of this period, the incubation was started by adding RuDP. The reaction mixture was incubated for 10 min at 25°. The reaction was stopped with 50 μl 6 N HOAc. A 10-μl aliquot was plated and counted.

Threose diphosphate final conc (M)	*Counts/min/10 µl aliquot*	*Percent inhibition*
0	16,000	—
$8 \cdot 10^{-6}$	15,000	6
$8 \cdot 10^{-5}$	14,000	15
$8 \cdot 10^{-4}$	7,600	53

ACKNOWLEDGEMENTS

The work described in this paper was sponsored in part by the United States Atomic Energy Commission and in part by the Department of Chemistry, University of California, Berkeley, Calif. (U.S.A.).

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