THE REDUCTIVE PENTOSE PHOSPHATE CYCLE III.[1] ENZYME ACTIVITIES IN CELL-FREE EXTRACTS OF PHOTOSYNTHETIC ORGANISMS

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The reductive pentose phosphate cycle (6, 29, 8) is currently believed to be the pathway by which CO_2 is converted to carbohydrate in photosynthesis. Evidence for the functional role of this cycle is derived mainly from isotope experiments. Brief exposure of photosynthesizing algae to $C^{14}O_2$ produces radioactive 3-phosphoglycerate (3-PGA)[3], labelled mainly in the carboxyl group (11). The cyclic nature of the photosynthetic carbon path is indicated by the subsequent appearance of isotopes in the α and β positions of 3-PGA, as well as in phosphate esters of ribulose and sedoheptulose (28). Kinetic studies by Calvin and Massini (14) and Bassham et al (7) on the fluctuation of pool sizes, revealed that transition from light to dark resulted in an increased concentration of 3-PGA accompanied by a decreased concentration of sugar diphosphate. Studies (43) carried out with algae subjected to changing partial pressures of CO_2 demonstrated a fall in 3-PGA and a rise in ribulose diphosphate (RDP) concentration when the CO_2 concentration was changed from 1 % to 0.003 %.

Enzymes of the pentose phosphate cycle are ubiquitously distributed (2, 4, 5, 17, 18, 20, 21, 22, 23, 24, 28, 29, 30, 36, 37, 38, 41). However, no single photosynthetic organism has been shown to possess all of the requisite enzymes and in amounts sufficiently high to support photosynthesis by this pathway. In this report, the quantitative aspects of all the pentose phosphate cycle enzymes in extracts of several photosynthetic organisms were investigated. We compared the rate of photosynthetic CO_2 fixation of the intact organism with the enzyme capacities (activities at saturating substrate concentration) in an extract of the same organism. Our results indicate that several enzymes of the pentose phosphate cycle appear to be insufficiently active to satisfy the rate of CO_2 fixation in the intact organism.

MATERIALS & METHODS

CULTURE OF ALGAE: A, Cultures of the thermophilic *Chlorella pyrenoidosa* Chick. strain 7-11-05 (33) were obtained from Dr. Dean Burk. The cells were grown in 500 ml gas wash bottles. Air containing 5 % CO_2 was bubbled through the bottles while they were gently shaken to keep the algae suspended. Illumination of 1,000 to 2,000 ft-c was supplied by a 150 w incandescent bulb placed about six inches from the culture bottles. At this site the temperature was maintained at 38° C. The medium was that recommended by Burk (personal communication). A liter of medium contained: $MgSO_4 \cdot 7H_2O$, 5.0 g; KH_2PO_4, 2.5 g; NaCl, 2.0 g; urea, 0.4 g; sequestrene-Na · Fe, 4.0 mg; $CaCl_2$, 22.0 mg; $CuSO_4 \cdot 5H_2O$, 0.158 mg; H_3BO_3, 5.7 mg; $MnCl_2 \cdot 4H_2O$, 3.6 mg; $ZnSO_4$, 0.44 mg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.035 mg; $NaVO_3$, 1.92 mg; the medium was adjusted to pH 5.7 with NaOH. The cells were harvested after growth overnight, washed at 3,000 × *g* at 20° C with 0.05 M tris buffer, pH 7.4. A pure culture of the same strain was also obtained from Dr. C. Sorokin and grown as described (34).

B, A slant of a low temperature *Chlorella pyrenoidosa* was kindly supplied by Dr. M. Gibbs. The cells were grown in 1 liter Erlenmeyer flasks in the medium of Norris et al (25), except that 10 mg NaCl per liter were added, and 5 mg $FeSO_4$ and 2 mg tartaric acid per liter were substituted for the suggested Fe-EDTA additions. The container was stoppered with a cotton plug through which a bubbling tube was inserted. Air containing 5 % CO_2 was bubbled through the medium under sterile conditions. The temperature was maintained at 23° and illumination at 1,000 ft-c. The cells were washed as described above.

C, A culture of *Euglena gracilis* Klebs., strain Z, was obtained from Dr. S. Hutner. The organisms were grown under sterile conditions in the medium of Cramer and Myers (16) at 24° C, and illumination was supplied by fluorescent lamps at an intensity of 400 ft-c. Cells were harvested after 6 days and washed at 1,000 × *g* at 2° C with 0.1 M tris, pH 7.4, containing 0.12 % EDTA.

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[3] Abbreviations: DPN = diphosphopyridine nucleotide. EDTA = ethylenediamine tetraacetate. FDP = fructose-1, 6-diphosphate. G-3-p = glyceraldehyde-3-phosphate. GSH = glutathione. 3-PGA = 3-phosphoglycerate. Pi = inorganic phosphate. R-5-p = ribose-5-phosphate. RDP = ribulose 1,5-diphosphate. SDP = sedoheptulose-1, 7-diphosphate. TPN = triphosphopyridine nucleotide. tris = tris (hydroxymethyl) aminomethane. Xu-5-p = xylulose-5-phosphate.

PREPARATION OF CELL-FREE EXTRACTS: A homogenate from fresh spinach leaves was prepared by the method of Arnon (3).

Extracts of Euglena and Chlorella were prepared in a Nossal shaker (26). Seven milliliters of 0.3 mm diameter glass beads (Minnesota Mining & Mfg. Co.) and the cell suspension in a volume of 10 ml were dispensed into the Nossal cup. The cup was evacuated (27) and shaken at 2° C for 10-second intervals, separated by 1 minute periods of cooling in an ice bath. Three shaking periods were sufficient to effect complete breakage of Euglena as indicated by microscopic observation. Four 10-second periods of shaking were used for Chlorella. After treatment in the Nossal shaker, the preparations were centrifuged for 10 minutes at 1,000 × *g* to free the suspensions of glass beads and cell debris.

REAGENTS: $Na_2C^{14}O_3$ solution was obtained from Tracerlab, Inc. Sequestrene–Na · Fe was obtained from the Alrose Chemical Co., Cranston, R.I. All other reagents and auxiliary enzymes were obtained as described previously (29, 30, 44, 15). Before use all barium salts were dissolved and passed through a Dowex-50 Na^+ column to remove the barium.

ASSAY OF ENZYMES, CO_2 FIXATION, & DETERMINATION OF CHLOROPHYLL: RDP carboxylase was determined either spectrophotometrically (29) or by a $C^{14}O_2$ fixation assay similar to that of Jakoby et al (24). In a volume of 0.5 ml, the following were added: tris buffer, pH 7.8, 20 μmoles; GSH, 2 μmoles; bovine serum albumin, 0.4 mg; $MgCl_2$, 4 μmoles; $KHC^{14}O_3$, 30 μmoles; RDP, 0.40 μmoles, and the extract to be assayed. After a suitable incubation period, 0.5 ml of 10 % trichloroacetic acid was added and an aliquot of the centrifuged supernatant solution was plated and counted.

Glyceraldehyde–3–P dehydrogenase and 3–phosphoglycerate kinase were assayed in the back direction (44). Transaldolase (15), xylulose–5–P epimerase and ribose–5–P isomerase (36), phosphoribulokinase (29), aldolase (44), glyceraldehyde–3–P isomerase (10), fructose diphosphatase, and sedoheptulose diphosphatase (30) were measured as described in the references. Transketolase was determined as previously (15) except that 10 μg of crystalline glyceraldehyde–3–P isomerase and α–glycerophosphate dehydrogenase were substituted for the 52 to 72 % ammonium sulfate fraction of rabbit muscle. All enzyme assays were carried out in either tris or glycylglycine buffer between pH 7.4 and 7.8.

Chlorophyll of spinach leaves and of Chlorella was determined by the method of Warburg (cf 39). Chlorophyll of Euglena and of spinach homogenate were determined by the method of Arnon (1). Rates of photosynthesis of intact cells were determined by fixation of radioactive bicarbonate. The experiments were carried out in a modified Warburg apparatus in which illumination was provided by a bank of fluorescent lamps mounted under the glass bottom of the tank providing 400 ft-c to the reaction vessels. This was increased to 1,000 to 2,000 ft-c by overhead illumination from 500 w incandescent bulbs. A cooling unit and heating bar provided a wide range of temperature control. The vessels were shaken in an air atmosphere.

RESULTS

CO_2 FIXATION OF INTACT ORGANISMS: Experiments were carried out with intact spinach leaf squares to ascertain the rate of CO_2 fixation obtainable in this tissue. Washed spinach leaves were destemmed and the midribs removed. The remainder of the leaf was cut into 1 cm squares. Three such squares were incubated in a 3 ml volume with NaCl (0.035 M), tris buffer (0.013 M, pH 7.4) and radioactive bicarbonate. Bicarbonate saturation was reached at 0.03 M. Experiments were carried out for 15 minutes at 20°. After terminating the experiment with 5 % trichloroacetic acid, the leaf squares and suspending medium were washed into a Potter-Elvejhem type homogenizer and ground to a uniform suspension. An aliquot of the suspension was transferred to a planchet, dried, and counted. With 30 μmoles bicarbonate per ml (about 10,000 counts/min per μmole) CO_2 fixation was 32 μmoles/mg chlorophyll/hour.

Washed Euglena were incubated with tris buffer (0.04 M, pH 7.4) and radioactive bicarbonate for the measurement of CO_2 fixation. The activity was relatively constant throughout a concentration range of bicarbonate of 1×10^{-3} M to 1.7×10^{-2} M. At higher concentrations, the rate of CO_2 fixation increased. The plateau region at the lower bicarbonate concentrations suggests that Euglena may have a concentrating mechanism for bicarbonate which is saturated at these levels. At a bicarbonate concentration of 1×10^{-2} M and illumination of 1,000 ft-c, the rate of CO_2 fixation at 20° was 100 μmoles/mg chlorophyll/hour. Under these conditions, the CO_2 fixation activity was light dependent, proportional to time and to the amount of Euglena added, at least up to a concentration of 0.3 mg chlorophyll/ml.

Washed cells of the thermophilic Chlorella, 7–11–05 (33) were incubated with radioactive $NaHCO_3$ at pH 7.4, tris 0.04 M. A light-dependent CO_2 fixation proceeding at a constant rate for at least one hour and proportional to cell concentration at low chlorophyll concentrations could be demonstrated. The carboxylation activity was saturated with bicarbonate at a concentration of 2×10^{-2} M. Rates of photosynthesis measured under these conditions ranged from 200 to 400 μmoles CO_2/mg chlorophyll/hour at 38° C. These rates, as determined by $C^{14}O_2$ fixation were found to be in satisfactory agreement with measurement of net CO_2 uptake.

The normal low temperature strain of *Chlorella pyrenoidosa* exhibited properties of CO_2 fixation essentially similar to that of the thermophilic organisms. At a bicarbonate concentration of 1×10^{-2} M and illumination of 1,000 ft-c the rate of CO_2 fixation was 150 μmoles/mg chlorophyll/hour at 23° C.

TABLE I

PENTOSE PHOSPHATE CYCLE ENZYME ACTIVITIES IN EXTRACTS OF PHOTOSYNTHETIC ORGANISMS

ENZYME	ACTIVITY (μmoles/mg CHLOROPHYLL/hr)*			
	SPINACH HOMOGENATE	EUGLENA	CHLORELLA (HIGH TEMP.)	CHLORELLA (LOW TEMP.)
3-PGA kinase	4,500	5,875	12,590	2,130
G-3-P isomerase	1,060	2,540	4,913	3,700
G-3-P dehydrogenase (DPN)	390	334	406	260
G-3-P dehydrogenase (TPN)	180	280	368	170
Aldolase	300	68	312	99
Phosphoribulokinase	600	107	1,133	227
Transketolase	300	98	214	73
R-5-P isomerase	508	169	664	196
Xu-5-P epimerase	1,630	360	1,584	22
RDP carboxylase	150	45	57	21
FDPase	39	13	21	22
SDPase	8	1.5	4	30
Transaldolase	6	4.5	1.2	1
CO_2 fixation in intact cells	32	100	259	150
Temp. ° C	20°	20°	38°	23°

*Calculated for temperature indicated in bottom line.

Fixation of CO_2 in the dark was well below 10 % of that in the light in spinach leaves, Euglena, and Chlorella.

ENZYME ACTIVITIES IN CELL-FREE EXTRACTS: In table I are listed representative activities of the pentose phosphate cycle enzymes in cell-free extracts of the four photosynthetic organisms examined. The enzyme capacities are expressed as μmoles of substrate converted to product in 1 hour by the amount of extract derived from organisms containing 1 mg chlorophyll. For comparative purposes all enzyme rates were corrected to the indicated temperature assuming a temperature coefficient of 2. It should be noted that in all cases the activities of RDP carboxylase, FDPase, SDPase, and transaldolase are lower than required to support the rate of CO_2 fixation of the whole cells. In spinach, only transaldolase and SDPase appear low.

Although some variations have been observed in the enzyme activities in extracts, e.g. from different batches of spinach, the recorded values are quite representative. For example, in about 12 assays of RDP carboxylase in various spinach samples variations of no more than a factor of 2 were encountered. Somewhat larger variations were noted in transaldolase activity which was too low for very accurate assays. Recovery experiments, e.g. with known transaldolase added to the crude extract, did not indicate the presence of an inhibitor.

It was conceivable that the method of preparing the extract resulted in incomplete release or destruction of enzyme activity. To explore this possibility a kinetic study of enzyme release was carried out (fig 1). A Chlorella suspension was assayed for two pentose phosphate cycle enzymes after successive disruption periods in the Nossal shaker. Three 10-second shaking periods released the maximum amount of RDP carboxylase and aldolase, while additional shaking periods neither released more enzyme activity nor resulted in the destruction of activity. Further-

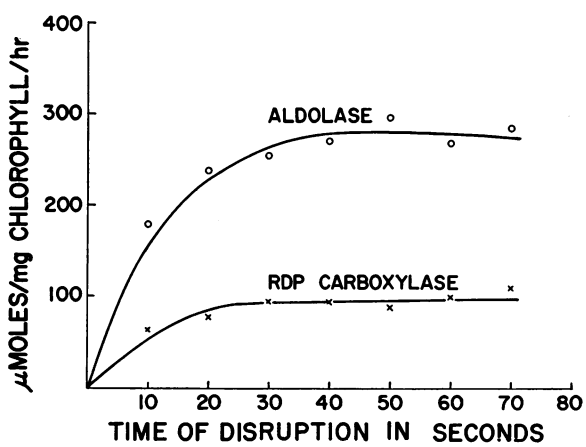

FIG. 1. Time course of enzyme release from chlorella 7-11-05 by disruption in a Nossal shaker. A suspension of Chlorella 7-11-05 was dispensed into a Nossal cup and subjected to 10 second periods of shaking. An aliquot was removed before shaking was begun and after each shaking interval. The samples were assayed for aldolase and RDP-carboxylase ($C^{14}O_2$ fixation assay). The assay temperatures were 26° C for aldolase and 38° C for RDP-carboxylase.

more, a small residue obtained after centrifugation of the disrupted Chlorella did not fix CO_2 nor did it stimulate the CO_2 fixing activity residing in the soluble fraction. Studies of the stability of the enzyme in the extract also failed to reveal any striking inactivation processes.

A closer examination was made of the RDP-carboxylase activity of Chlorella in order to determine whether or not the addition of certain cofactors might result in a marked stimulation of its activity. The addition of the growth medium, or of cobalt, nickel, and manganese, which can substitute for magnesium in spinach RDP-carboxylase (42), were ineffective in Chlorella extract. The enzyme did not exhibit any unusual temperature properties since it had a typical Q_{10} of 2.

A study of the effect of bicarbonate concentration on the RDP-carboxylase activity of Chlorella extract revealed that the activity was being measured at a bicarbonate concentration which resulted in maximal activity. Note that the curve was essentially the same as that obtained in a bicarbonate concentration study for photosynthetic CO_2 fixation of the intact organisms.

The possibility of a transcarboxylase activity of RDP-carboxylase (29) was tested by addition of various potential CO_2 donors. None of a number of intermediates of the urea cycle (ornithine, citrulline, arginine) or Krebs cycle (oxaloacetate, succinate, malate, citrate, isocitrate, glutamate) stimulated CO_2 fixation by the extract in the presence of RDP. None of the factors tested appeared to catalyze appreciable CO_2 fixation in the absence of RDP. ATP, which has previously been observed to inhibit purified spinach RDP-carboxylase (unpublished observations in this laboratory), resulted in a 35 % inhibition of the activity in the crude Chlorella extract.

A survey test of some vitamins, concentrated cofactors from liver or yeast and crude boiled juice of Chlorella revealed no stimulation of the carboxylation of RDP. In all cases, the omission of RDP resulted in no CO_2 fixation.

Discussion

Our studies show that, as with glycolysis (44) and the urea cycle (13), assays for the pentose phosphate cycle enzymes may be applied to crude cell extracts. In analyses of glycolysis, the urea cycle, and pyrimidine biosynthesis, the least active enzyme had a capacity several-fold higher than had the overall process (13, 44–46). On the basis of the observation that cell-free extracts of *Anacystis nidulans* show low aldolase activity, Richter (31) has concluded that the pentose phosphate cycle is not the pathway for hexose synthesis by this organism. Similar observations have been made by Szymona and Doudoroff (35) in a study of the metabolism of *Rhodopseudomonas spheroides*. In the present study of the reductive pentose phosphate cycle, the activities of several enzymes (RDP–carboxylase, fructose diphosphatase, sedoheptulose diphosphatase, transaldolase) are not high enough to satisfy the rate of overall CO_2 fixation observed in intact cells. It should be emphasized that the conditions employed in this work of measuring CO_2 fixation in intact cells in tris buffer are rather unphysiological, particularly in the case of spinach leaves. The conditions were chosen only to allow for a direct comparison with the enzyme assays. The relatively low values of photosynthesis obtained with intact spinach leaves represents therefore, a minimum value. Any increase in the rate of CO_2 fixation under more physiological conditions would make the discrepancy with the enzyme assays even greater.

Of the four enzymes that were found particularly low, only transaldolase was measured in the direction opposite to that believed to be operative in photosynthesis. This choice of assay procedure was determined only by the limited availability of erythrose–4–P. In view of the particularly low values for transaldolase observed in extracts of photosynthesizing cells, a few measurements in Chlorella extracts were carried out in the other direction, measuring the formation of glyceraldehyde–3–P from fructose–6–P and erythrose–4–P. Although the reaction proceeded faster in this direction (3–6 μmole/mg chlorophyll/hr) it still represented only a small fraction of the overall CO_2 fixation rate. To eliminate the possibility that an inhibitor in the extract interfered with the assay of these enzymes of low activity, known amounts of purified enzyme solutions were added to the crude extract and reassayed. No evidence for an inhibition was obtained since the recovery of activities was satisfactory.

It is obvious that this type of data, obtained after disruption of the cell structure, is insufficient to eliminate the reductive pentose phosphate cycle as a major CO_2 fixation pathway in photosynthesizing cells. They do, however, suggest that a search should be made for alternative enzymes catalyzing individual steps of the cycle, particularly where apparent enzyme deficiencies exist. For example, a phosphate transfer reaction might substitute for the hydrolytic cleavage of fructose diphosphate to fructose–6–P. Attempts to demonstrate such a reaction with various P–acceptors, such as ribulose–5–P, have been made in our laboratory during the past years without success. For the utilization of erythrose–4–P in the cycle, the transaldolase reaction could be replaced by aldolase followed by a sedoheptulose diphosphatase (32, 9). Since the latter activity is also low in extracts of photosynthesizing cells, phosphate transfer to ribulose–5–P from sedoheptulose diphosphate was explored, again with no success.

An even more difficult problem is the low activity of RDP–carboxylase. It has been pointed out previously (29) that its low affinity for $KHCO_3$ calls for a large excess of this enzyme in photosynthesizing cells. The low RDP-carboxylase content reported in this study in comparison to the overall CO_2 fixation rate further accentuates the discrepancy between enzyme activity and CO_2 fixation rate of intact cells.

In view of this low affinity of carboxylase for CO_2 several investigators have considered the possibility that an activated form of CO_2 is involved in photosynthesis. Since crude extracts were shown to require the same high concentration of bicarbonate for maximal CO_2 fixation into RDP as the purified enzyme (29) we compared the effect of bicarbonate concentration of CO_2 fixation in intact cells and crude extracts (table II). In spinach and Chlorella, the bicarbonate concentration required for saturation in the extract coincides with that in the whole cells. The lower bicarbonate concentration required for saturation of CO_2 fixation in intact Euglena (compared to Euglena extract) may be due to a CO_2 concentrating system. Except for Euglena, therefore, the data speak against an activated form of CO_2 participating in photosynthesis.

Although attempts have been made and described above to rule out enzyme inactivation during the process of mechanical shaking, an alternative procedure of cell disruption, namely sonic disintegration, was employed in some experiments. Breakage of Chlorella was complete after 20 minutes of sonic vibration. Low speed centrifugation of the suspension resulted in almost no residue and was therefore omitted. Such crude suspensions must therefore have included broken cell wall material and any enzymes associated with it. Nevertheless, enzyme assays of RDP-carboxylase, fructose diphosphatase, glyceraldehyde-3-P dehydrogenase, and transaldolase gave values similar to those found in extracts obtained in the Nossal shaker.

The possibility that the pentose phosphate cycle enzymes within the intact cell operate in an organized way, resulting in an increased activity, is not one that is easily tested with the techniques currently available. The measurement of the enzymes in the profiles reported here were carried out at optimal substrate concentrations. Since it is unlikely that such conditions are prevalent in the intact cell, the discrepancy discussed above may be even greater than is immediately apparent from the data.

Numerous other CO_2 fixation pathways have been proposed in the past, particularly some channeling into amino acids (19, 40). However, none of these pathways has been formulated to include a cyclic regeneration of a CO_2 acceptor, a feature which is present in the reductive pentose phosphate cycle. A scheme lacking in this essential point fails to account for the process of photosynthesis.

TABLE II

COMPARISON OF SATURATING CO_2 CONCENTRATIONS IN DIFFERENT TISSUES & EXTRACTS

SYSTEM EXAMINED	SATURATING BICARBONATE CONC (M)
Spinach leaves	3×10^{-2}
Spinach homogenate	3×10^{-2}
Euglena	1×10^{-3}
Euglena extract	3×10^{-2}
Chlorella 7–11–05	2×10^{-2}
Chlorella extract	2×10^{-2}

SUMMARY

Profiles of the enzymes of the pentose phosphate cycle were determined in cell-free extracts of spinach, Chlorella and Euglena. A comparison of these activities with the rate of photosynthetic CO_2 fixation of intact cells revealed marked discrepancies. Certain enzymes of the pentose phosphate cycle appeared to be present in insufficient amounts to support the rate of photosynthesis of the intact cells. Evidence was presented suggesting that the enzymes were completely extracted and not inactivated in the process of preparing cell-free extracts. The significance of these findings is discussed.

ACKNOWLEDGMENTS

The authors wish to thank Mrs. E. Schroeder for her valuable technical assistance, and Mr. Philip Schmidt of Farmingdale, Long Island, and Mr. Walter Sapanski who supplied the fresh-cut spinach used in these experiments.

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