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Reductive Pentose Phosphate Cycle and Oxidative Carbohydrate Metabolic Activities in Pea Chloroplast Stroma Extracts[1]

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ABSTRACT

Oxidative and reductive carbohydrate metabolism was studied in reaction mixtures based on chlorophyll-free stromal extracts from chloroplasts of *Pisum sativum*. A new assay system for the reductive pentose phosphate cycle was characterized.

When provided with ATP, an enzymic ATP-regenerating system and reduced pyridine nucleotide, substantial rates of CO_2 fixation and pyridine nucleotide oxidation were observed following the addition of millimolar concentrations of reductive pentose phosphate cycle intermediates. The reduced pyridine nucleotide requirement could be met either by NADPH, or by NADH plus the added enzymes NAD^+-glyceraldehyde phosphate dehydrogenase and phosphoglycerate kinase. When the assay system was primed with small amounts of reductive pentose phosphate cycle intermediates, lower rates of pyridine nucleotide oxidation were observed, but turnover of the complete cycle was demonstrated. Autocatalytic effects were not evident. The optimum pH and Mg concentrations for cycle turnover were similar to those believed to exist in the stroma of intact chloroplasts in the light.

Oxidative carbohydrate metabolism was studied by supplying oxidized pyridine nucleotide and measuring its rate of reduction in the presence of sugar phosphates. Glycolytic activity, estimated as the rate of fructose-6-phosphate entry to the phosphofructokinase reaction was 2.7 micromoles per milligram chlorophyll per hour when fructose-6-phosphate was provided as substrate. Evidence based on glucose-6-phosphate and ribose-5-phosphate-dependent $NADP^+$ reduction showed that the oxidative pentose phosphate cycle was also active. Apparent oxidative pentose phosphate cycle turnover in the presence of ribose-5-phosphate, estimated as the rate of glucose-6-phosphate entry to the glucose-6-phosphate dehydrogenase reaction, was 1.7 micromoles per milligram chlorophyll per hour.

It was concluded that under the defined conditions, reductive pentose phosphate cycle activity could be measured without interference from oxidative carbohydrate metabolism in this experimental system.

Much information has been gained on the regulation of photosynthetic CO_2 fixation from *in vitro* studies based on three types of experimental systems; (*a*) isolated intact chloroplasts (22), (*b*) reconstituted chloroplasts (17), and (*c*) the assay of individual enzymes (1, 10). Reconstituted chloroplasts offer the possibility of studying turnover of the entire reductive pentose phosphate cycle (5, 23) in a system free of the compartmentation effects imposed by the envelope membranes of intact chloroplasts. The reconstituted chloroplast system is not, however, free of drawbacks. Due to the dilution (by a factor of 500 or more) which occurs when the chloroplast envelopes are ruptured during the preparation procedure, it is necessary to add ADP (or ATP) and $NADP^+$ to the final reaction mixture (17), with the result that the amounts of these compounds present are much higher on a Chl basis than *in vivo*. The biochemical activities observed may consequently be artificially constrained, particularly if the ATP/ADP ratio is low (15), by the available rates of photophosphorylation and electron transport unless thylakoids of very high activity are used (24). Additionally, the study of redox-related enzyme activation effects may be complicated by variations in redox potential caused by changes in the rate of electron flow in the thylakoids.

A new experimental system, based on Chl-free stromal extracts and capable of exhibiting reductive PPC[4] turnover is characterized in this paper. In this system the thylakoids were omitted and NADPH or NADH and ATP were supplied in concentrations approximating those of the stroma *in vivo* (17). The concentration of ADP was kept low by the addition of phosphocreatine and creatine kinase, and the progress of reactions was monitored by either measuring pyridine nucleotide oxidation or CO_2 fixation. This system has already been used to show that the activity of the reductive PPC is inhibited by Pi (6). The reduction of 3-P glycerate (15) and carboxylation of ribulose-1,5-bisP (14) in similar systems have previously been investigated.

Reaction mixtures based on chloroplast stromal extracts have been used to study the oxidative metabolism of sugar phosphates (7). These activities, involving pyridine nucleotide reduction, obviously have the potential to interfere with measurements of the reductive PPC based on NADPH or NADH oxidation. Accordingly, the oxidative carbohydrate metabolic activities have been characterized here for comparison with the reductive PPC activities.

MATERIALS AND METHODS

Peas (*Pisum sativum*, Massey Gem, Arthur Yates & Co., Sydney, Australia) were grown in vermiculite for 9 to 11 days in a glasshouse under natural lighting conditions.

Pea leaves (50 g) were harvested, washed in distilled H_2O and disrupted by a Polytron blender (3 s) in 200 ml Medium 1 containing 0.33 M glucose, 500 mM Na_2HPO_4, 50 mM KH_2PO_4, 5 mM $MgCl_2$, 11 mM NaCl, 7.5 mM sodium isoascorbate, adjusted to pH 6.5 with KOH. The chloroplasts were pelleted by centrifuging for 30 s at 2,500*g*. Following resuspension in 100 ml Medium 2 containing 0.33 M sorbitol, 2 mM EDTA, 1 mM $MgCl_2$, 0.2% BSA, 0.5 mM DTT, 50 mM Hepes adjusted to pH 8.0 with KOH, and centrifugation for 30 s at 2,500*g*, the chloroplasts were resuspended in 2 ml Medium 2. The resulting suspension usually contained 90–95% intact chloroplasts measured by ferricyanide reduction (13).

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[4] Abbreviations: Pi, inorganic phosphate; PPC, pentose phosphate cycle.

For the preparation of chloroplast extract, chloroplasts were osmotically shocked by resuspension in a 1/13 dilution of Medium 2 and centrifuged for 15 min at 9,000*g*. The resulting pellet of envelope-free chloroplasts was retained for Chl estimation (26). The supernatant, (chloroplast stromal extract), was subjected to concentrating dialysis (MicroProDiCon, Biomolecular Dynamics, OR). A 10,000 mol wt cut-off membrane was used and the chloroplast extract dialyzed against 1 liter of 10 mM Hepes, 10 mM KCl, 1 mM EDTA and 15 mM $MgCl_2$, adjusted to pH 8.0 with KOH. In addition, the dialysis medium contained 0.5 mM DTT when the chloroplast extract was to be used for reductive PPC assays. The concentrating dialysis was maintained for 4 h at 2 C and the volume of the chloroplast extract was reduced by a factor of 4. The concentrated chloroplast extract was immediately subdivided into 250 μl aliquots in small plastic tubes and placed in liquid N_2. Assays on a rethawed sample of chloroplast extract were completed within 2 h. Activity loss under these conditions was less than 1% per day for all measured activities. Protein was estimated by the Biuret method (11).

Pyridine nucleotide oxidation or reduction was measured spectrophotometrically (Varian 635) at 25 C. Each reaction mixture contained 40 mM Hepes, 8 mM KCl, 0.8 mM EDTA, chloroplast extract (1.3–2.7 mg protein) and other reagents as specified in a total volume of 500 μl at pH 8.0.

For the assay of reductive PPC activities, each reaction mixture contained, additionally, 20 mM $MgCl_2$, 10 mM DTT, 4 mM ATP, 10 mM phosphocreatine, 1.1 units creatine kinase (EC. 2.7.3.2), 10 mM $NaHCO_3$ and either 1 mM NADPH, or 1 mM NADH plus 1.25 units of NAD^+-glyceraldehyde-P dehydrogenase (EC. 1.2.1.12) and 1.7 units of phosphoglycerate kinase)EC. 2.7.2.3) from yeast. Before commencing each assay the chloroplast extract was preincubated in 20 mM $MgCl_2$, 10 mM $NaHCO_3$ and 10 mM DTT for 5 min at 25 C.

For the assay of oxidative sugar phosphate metabolic activities, each reaction mixture contained, additionally, 2 mM $MgCl_2$, 2 mM ATP, 2 mM ADP, 10 mM Pi and either 1 mM $NADP^+$, or 1 mM NAD^+ plus 1.25 units of NAD^+-glyceraldehyde-P dehydrogenase and 1.7 units of phosphoglycerate kinase from yeast.

Biochemicals were purchased from the Sigma Chemical Company and were all of high purity, except for erythrose-4-P (80%) and sedoheptulose-7-P (85%) which was supplied as the barium salt, and was converted to the sodium salt by passage through a small column of ion-exchange resin (Na^+ amberlite IR—20) before use.

RESULTS

REDUCTIVE PENTOSE PHOSPHATE CYCLE ACTIVITY

Substantial rates of pyridine nucleotide oxidation were observed following the addition of reductive PPC intermediates to reaction mixtures (Table 1). The rates with NADH plus added yeast phosphoglycerate kinase and NAD^+-glyceraldehye-P dehydrogenase were always equal to or greater than those observed with NADPH (results not shown). The NADH-linked system was therefore subsequently utilized for all experiments since its high 3-P-glycerate reducing activity (Table I) ensured that this reaction would not be rate-limiting when other substrates were provided.

When fructose-1,6-bisP was provided as substrate (Fig. 1) the amount of CO_2 fixed corresponded to half the amount of NADH oxidized, as reported previously for ribose-5-P and ribulose-1,5-bisP in similar reaction mixtures (14). When primed with small amounts of 3-P-glycerate, either alone or with erythrose-4-P, there was a rapid initial oxidation of an equimolar amount of NADH without any CO_2 fixation. Following this, CO_2 fixation commenced and was then accompanied by further NADH oxidation with a stoichiometry of $1CO_2$ fixed/2 NADH oxidized.

The rate of CO_2 fixation in C_3 plants in air under normal conditions is about 100 μmol CO_2 mg^{-1} Chl h^{-1} (16), or 200 μmol pyridine nucleotide oxidized mg^{-1} Chl h^{-1}. This rate was substantially exceeded when 2 mM ribulose-1,5-bisP was added to reaction mixtures since CO_2 was present, in this case, at saturating levels (10 mM $NaHCO_3$). This rate was also exceeded with ribose-5-P as substrate and was approached with sedoheptulose-7-P. Progressively lower activities were observed with cycle intermediates further removed around the reductive PPC from the ribulose-1,5-bisP carboxylase reaction. The rates with sedoheptulose-7-P and fructose-6-P were increased substantially by the inclusion of small amounts of dihydroxyacetone-P which presumably provided, after conversion to glyceraldehyde-3-P, the co-substrate necessary for sedoheptulose-7-P and fructose-6-P to enter the transketolase reactions. The rate of NADH oxidation with sedoheptulose-1,7-bisP considerably exceeded that with fructose-1,6-bisP, consistent with the former intermediate leading to the generation of twice as much pentose phosphate as the latter by the cycle reactions. Erythrose-4-P and dihydroxyacetone-P were less effective substrates for NADH oxidation although, with dihydroxyacetone-P, almost complete cycle turnover was occurring at a rate about 10% of that *in vivo*, because glyceraldehyde-3-P is the product of NADH oxidation. Linear rates were observed following priming with small (100 nmol) amounts of dihydroxyacetone-P, which persisted during the oxidation of at least 400 nmol NADH, thus demonstrating cycle turnover (5). Turnover of the complete reductive PPC also occurred following the initial rapid NADH oxidation resulting from the addition of small amounts of 3-P-glycerate (Fig. 1 and Table I). The linear time courses of NADH oxidation with dihydroxyacetone-P or 3-P-glycerate as primer suggest that autocatalytic effects (23) were negligible in this system.

Reductive PPC turnover, primed by small amounts of dihydroxyacetone-P, was substantially affected by Mg concentration with a sharp optimum at 17.5 mM Mg (Fig. 2). At all Mg concentrations tested, the initial rate of NADH oxidation in the presence of small amounts of 3-P-glycerate was much higher than with dihydroxyacetone-P, showing that effects of Mg on the

Table I. *Rates of NAD(P)H Oxidation with Reductive Pentose Phosphate Cycle Metabolites as Substrates*

All rates were determined with the same chloroplast extract using the NADH-linked reductive pentose phosphate assay with 2 mM concentration of each substrate except where otherwise stated. The chloroplast extract was prepared from chloroplasts which contained 21.1 mg stromal protein/mg Chl.

Substrate	Oxidation Rates	
	μmol mg^{-1} protein h^{-1}	*μmol mg^{-1} Chl h^{-1}*
Ribulose-1,5-bisP	25.87	546
Ribose-5-P	21.45	453
Sedoheptulose-7-P	8.72	184
Sedoheptulose-7-P plus dihydroxyacetone-P (0.2 mM)	12.79	270
Sedoheptulose-1,7-bisP	5.31	112
Fructose-6-P	3.68	77.6
Fructose-6-P plus dihydroxyacetone-P (0.2 mM)	6.74	142
Fructose-1,6-bisP	3.51	74.1
Erythrose-4-P	2.41	50.9
Erythrose-4-P plus dihydroxyacetone-P (0.2 mM)	2.64	55.7
Dihydroxyacetone-P	2.51	53.0
Dihydroxyacetone-P (0.2 mM)	1.07	22.6
3-P-Glycerate (0.2 mM, following initial rapid oxidation)	0.72	15.2
3-P Glycerate	161.5	
3-P Glycerate (NADPH-linked assay)	56.3	1188

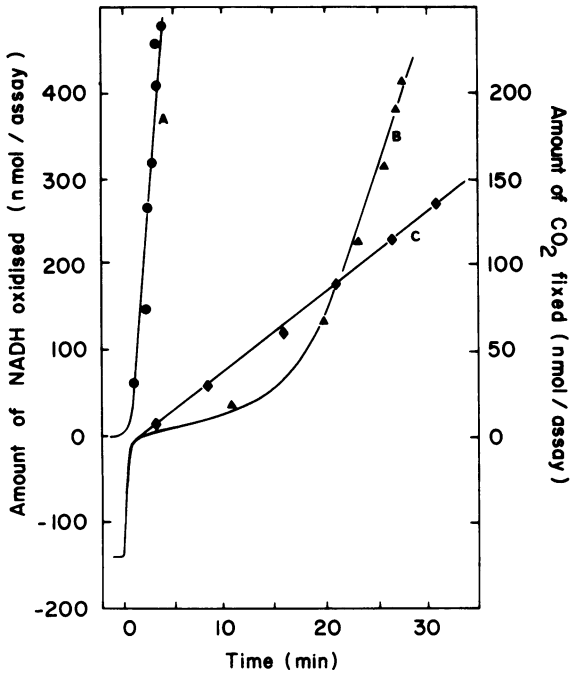

FIG. 1. Simultaneous measurement of CO_2 fixation and NADH oxidation following the addition of 1 μmol fructose-1,6-bisP (●, trace A, respectively), 1 μmol erythrose-4-P plus 140 nmol 3-P-glycerate (▲, trace B) and 140 nmol 3-P-glycerate (◆, trace C). Traces B and C have been displaced downwards by the equivalent of 140 nmol NADH for comparative purposes. The volume of the reaction mixture was 750 μl and [^{14}C]sodium bicarbonate (0.1 Ci mol^{-1}) was used; all assay conditions otherwise as for NADH-linked reductive pentose-P assay "Methods." Carbon fixation was measured by withdrawing 20-μl samples of each reaction mixture at intervals and injecting into 100 μl of methanol in a scintillation vial. Formic acid (10 μl) was then added and the contents of the vial evaporated to dryness before the addition of scintillation cocktail. Radioactivity was determined in a Packard C-2425 scintillation counter. Control samples, for measuring total radioactivity, were taken at the commencement and conclusion of each experiment by injecting 20-μl samples into scintillation vials containing 10 μl Carbosorb 2 (Packard) in addition to scintillation cocktail. Quench compensation was performed by the internal standard method.

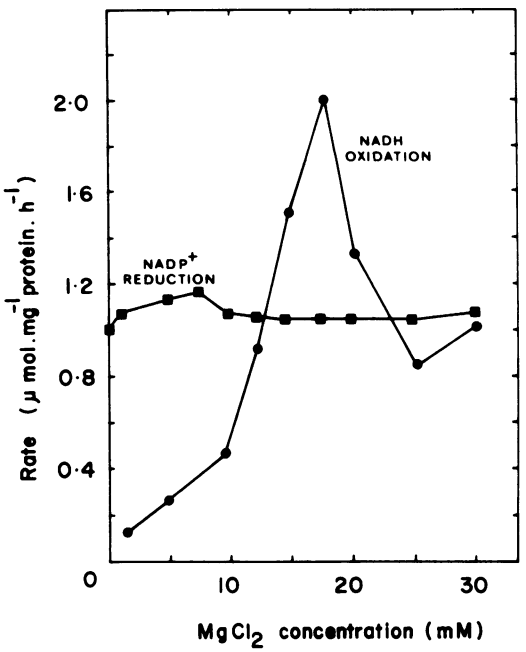

FIG. 2. Effects of Mg concentration. Rates of NADH oxidation following the addition of 100 nmol dihydroxyacetone-P to reductive pentose-P assay mixtures (●), and rates of NADP$^+$ reduction following the addition of 1 μmol glucose-6-P to oxidative sugar phosphate assay mixtures (■).

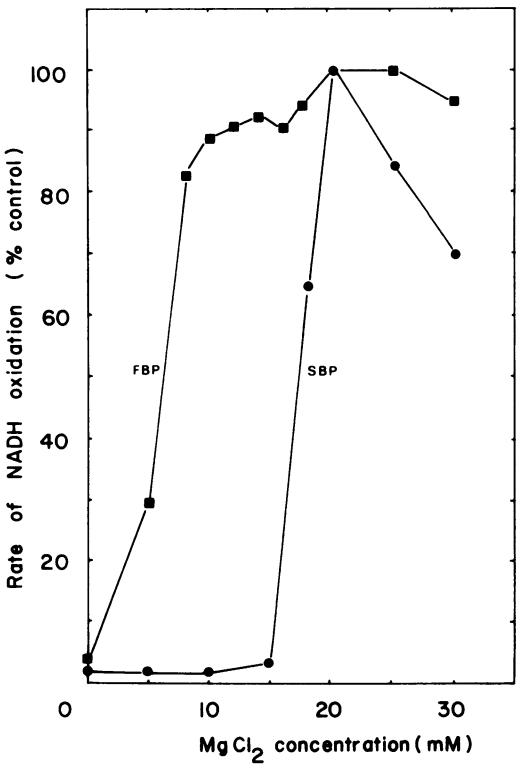

FIG. 3. Effects of Mg on the rates of NADH oxidation in reductive pentose-P assay mixtures with 1 μmol fructose-1,6-bisP (■; control rate, 20 mM Mg, 3.08 μmol mg^{-1} protein h^{-1}), or with 1 μmol sedoheptulose-1,7-bisP (●; control rate, 20 mM Mg, 1.62 μmol mg^{-1} protein h^{-1}).

coupling enzymes did not make a significant contribution towards the effect seen in Figure 2. The fructose- and sedoheptulose-bisphosphatases are believed to be primarily responsible for the regulation of carbon fixation by the stromal Mg concentration in intact chloroplasts (18). It is, therefore, interesting to compare the effects of Mg on NADH oxidation with substrate levels of fructose-1,6-bisP and sedoheptulose-1,7-bisP (Fig. 3). Whereas fructose-1,6-bisP-dependent NADH oxidation exhibited a broad response with near-maximum rates in the range 10–30 mM Mg, that with sedoheptulose-1,7-bisP showed a sharp optimum at 20 mM Mg and very low activity at 15 mM Mg. The apparent inconsistency between this and the response of dihydroxyacetone-P-primed reductive PPC turnover, which exhibited substantial activity at 15 mM Mg, may be due to the extra Mg binding capacity of the 2 mM sedoheptulose-1,7-bisP. This suggests that the Mg requirements of the complete reductive PPC may be dictated largely by the Mg requirements of sedoheptulosebisphosphatase, the enzyme responsible for the utilization of sedoheptulose-1,7-bisP. Determinations of free Mg^{2+} in these reaction mixtures by the Eriochrome Blue spectrophotometric method (9) showed that in the presence of 20 mM added Mg, the concentration of unbound Mg^{2+} was 5.5 mM.

The pH-dependency of the reductive PPC was examined in reaction mixtures exhibiting cycle turnover after priming with dihydroxyacetone-P (Fig. 4). There was a marked response to pH with maximum activity at pH 8.0. Werdan *et al.* (25) have previ-

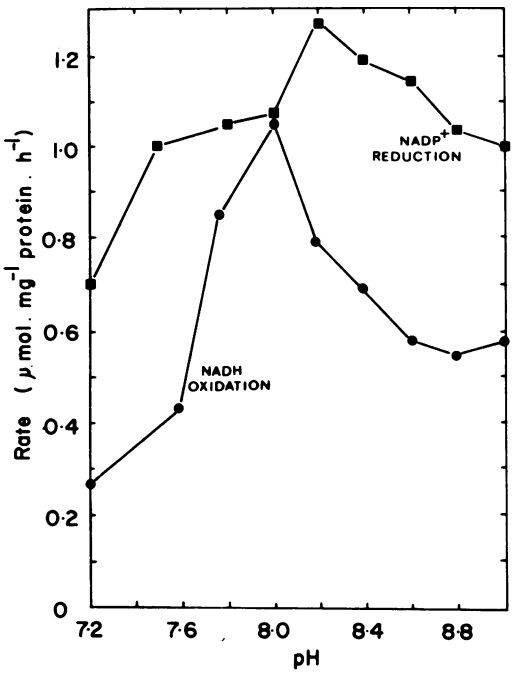

FIG. 4. Effects of pH. Rates of NADH oxidation following the addition of 100 nmol dihydroxyacetone-P to reductive pentose-P assay mixtures (●), and rates of $NADP^+$ reduction following the addition of 1 μmol glucose-6-P to oxidative sugar phosphate assay mixtures (■).

Table II. *Comparison of Rates of $NADP^+$ and NAD^+ Reduction with Different Substrates*

Each substrate was added (final concentration, 2 mM) to oxidative sugar phosphate assay mixtures containing either $NADP^+$ or NAD^+ plus the yeast enzymes 3-P-glycerate kinase and NAD^+-glyceraldehyde-3-P dehydrogenase. Initial reduction rates remained linear for at least 10 min except with ribose-5-P. The chloroplast extract was prepared from chloroplasts which contained 23.9 mg protein/mg Chl.

Substrate	Reduction Rates	
	$NADP^+$	NAD^+
	nmol mg^{-1} protein h^{-1}	
Fructose-6-P	955	229
Glucose-6-P	1,077	194
6-P-Gluconate	1,055	0
Ribose-5-P (initial)	687	1,640
Ribose-5-P (after 5 min)	143	0
Fructose-1,6-bisP	944	4,535

ously demonstrated an almost identical response of CO_2-fixation by illuminated spinach chloroplasts to the pH in the stroma.

OXIDATIVE SUGAR PHOSPHATE METABOLIC ACTIVITIES

Glucose-6-P-dependent $NADP^+$ reduction was inhibited by DTT, a known inhibitor of glucose-6-P dehydrogenase (2), and was relatively insensitive to ATP and ADP concentrations (results not shown) as reported previously (7). In contrast to the reductive PPC, this activity was insensitive to Mg concentration under the conditions of assay (Fig. 2) and displayed a broad response to pH with maximum activity at pH 8.2 (Fig. 4).

$NADP^+$ was reduced at much higher initial rates than NAD^+ with fructose-6-P, glucose-6-P and 6-P-gluconate as substrates (Table II). Conversely, higher initial rates were observed for NAD^+ reduction with ribose-5-P and fructose-1,6-bisP. The complete absence of 6-P-gluconate-dependent NAD^+ reduction showed that no oxidative PPC activity occurred with the NAD^+-linked system. Additionally, plant glucose-6-P dehydrogenase is $NADP^+$-specific (3). The NAD^+ reduction observed with glucose-6-P and fructose-6-P must therefore represent utilization via a glycolytic sequence (Fig. 5).

Fructose-1,6-bisP utilization was very rapid with the NAD^+-linked system (Table II) and was therefore not rate limiting in the glycolytic sequence. It may be assumed that 2 NAD^+ were reduced for each molecule of fructose-6-P that entered the phosphofructokinase reaction (Fig. 5). Half of the observed rate of fructose-6-P-dependent NAD^+ reduction (Table II), expressed on a Chl basis, is 2.7 μmol mg^{-1} Chl h^{-1}. This may be compared with recent direct enzymic estimates of phosphofructokinase activity of 6.5 μmol mg^{-1} Chl h^{-1} for pea chloroplasts (20), and of 2.5 μmol mg^{-1} Chl h^{-1} for spinach chloroplasts (8).

The rate of $NADP^+$ reduction with fructose-6-P as substrate was more than four times that for NAD^+ (Table II), even though fructose-1,6-bisP-dependent NAD^+ reduction was much faster. The extra fructose-6-P-dependent $NADP^+$ reduction must be due to oxidative PPC activity. Further evidence for oxidative PPC activity was provided by the observed ribose-5-P-dependent pyridine nucleotide reduction (Table II). The initial rapid rate of NAD^+ reduction resulted from the conversion of ribose-5-P to glyceraldehyde-3-P and fructose-6-P by part of the oxidative PPC, followed by glycolytic conversion of these intermediates to 3-P-glycerate (Fig. 5). Turnover of the oxidative PPC cannot occur under these conditions, due to the aforementioned $NADP^+$-specificity of the glucose-6-P and 6-P-gluconate dehydrogenases. The rate of NAD^+ reduction fell to zero within 5 min, showing that the extent of ribose-5-P utilization was very limited. The free energy change for this entire reaction sequence from ribose-5-P to 3-P-glycerate is small ($\Delta G' = -2.5$ kcal [4]), and equilibration after accumulation of some 3-P-glycerate and other products would be expected.

Although the initial rate of ribose-5-P-dependent $NADP^+$ reduction also was not maintained, the rate established after 5 min was linear and persisted until more than 50% of the $NADP^+$ present was reduced. This sustained $NADP^+$-specific reduction with ribose-5-P as substrate must result from the oxidative PPC

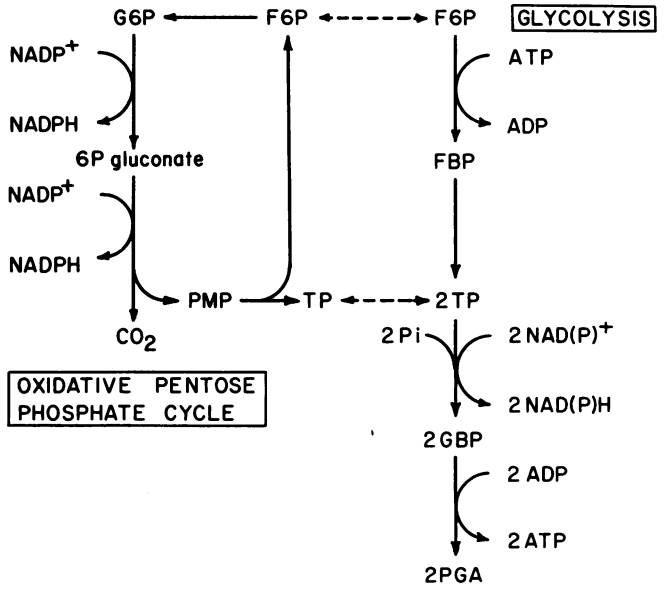

FIG. 5. Abbreviated scheme for oxidative carbohydrate metabolism in chloroplast extracts, showing the interactions between glycolysis and the oxidative pentose-P pathway (FBP: fructose-1,6-bisP, F6P: fructose-6-P, GBP: glycerate-1,6-bisP, G6P: glucose-6-P, PMP: pentose mono-P, TP: triose-P).

reactions progressing to the formation of glucose-6-P and its subsequent oxidation, and represents complete or near-complete oxidative PPC activity. If it is assumed that two $NADP^+$ are reduced per glucose-6-P utilized in the cycle, then the sustained ribose-5-P-dependent $NADP^+$ reduction (Table II) represents a rate of glucose-6-P oxidation of 1.7 μmol mg^{-1} Chl h^{-1}.

The inclusion of 10 mM Pi in the oxidative carbohydrate assay mixtures was intended to reproduce the high Pi concentrations in the stroma of intact chloroplasts in the dark (12). Although Pi is an inhibitor of the reductive PPC (6), it was without effect on oxidative PPC activity (glucose-6-P-dependent $NADP^+$ reduction) and resulted in some stimulation of fructose-1,6-bisP-dependent pyridine nucleotide reduction.

DISCUSSION

These results show that reductive PPC turnover can be demonstrated in reaction mixtures based on chloroplast stromal extracts but free of thylakoids. This "dark" CO_2 fixation exhibited a stoichiometry of 2 NADH oxidized per CO_2 fixed in the presence of a variety of reductive PPC intermediates. Pyridine nucleotide oxidation seems, therefore, to be a reliable measure of CO_2 fixation in this system and provides a convenient spectrophotometric assay. The P-creatine/creatine kinase system, by maintaining high ATP:ADP ratios, biased the phosphoglycerate kinase reaction strongly towards glycerate-1,3-bisP formation (19). This ensured that the conversion of 3-P-glycerate to glyceraldehyde-3-P was rapid and complete (15).

The measured reductive PPC activity exhibited responses to Mg concentration and pH similar to that of CO_2 fixation in intact chloroplasts (22). In a previous study (15) of 3-P-glycerate reduction by spinach chloroplast extracts in reaction mixtures similar to that used here, no evidence was found of appreciable reductive PPC activity, a result that can now be attributed to the use of suboptimal pH and Mg concentration. Glucose-6-P-dependent $NADP^+$ reduction, however, was relatively much less sensitive to Mg concentration and pH, suggesting that other factors may be of more importance in any dark/light regulation of oxidative PPC activity.

The rate of sedoheptulose-1,7-bisP-dependent NADH oxidation in this system was equivalent to about half the *in vivo* rate, and was greater than that with fructose-1,6-bisP (Table I). Sedoheptulose-bisphosphatase is regarded as the only enzyme of the reductive PPC for which the measured enzymic activity is apparently too low to support the observed *in vivo* rate of CO_2 fixation (10). In fact, the *in vivo* reductive PPC activity results from the sum of the fructose- and sedoheptulose-bisphosphatase activities since these two reactions operate in parallel, rather than in series, in terms of regeneration of pentose-P.

When small amounts of 3-P-glycerate or dihydroxyacetone-P were added to reductive pentose-P assays as primer, the resultant rates of CO_2 fixation and NADH oxidation were linear. Had significant autocatalysis been present, an increase in these rates with time should have occurred. In the experiment of Figure 1C, for example, a linear rate of CO_2 fixation was maintained for 30 min after priming the system with 140 nmol 3-P-glycerate. During this period, the amount of carbon fixed (about 140 nmol) increased the total fixed carbon pool in the assay mixture by only one third. Since this carbon pool was distributed among the many intermediates of the reductive PPC, the steady-state concentration of ribulose-1,5-bisP would be expected to remain low, and this was reflected by the relatively low rates of CO_2 fixation observed. It would probably be necessary to obtain several-fold increases in the total pool of fixed carbon during the course of the assay before autocatalysis could be demonstrated. Autocatalysis is readily demonstrated with intact isolated chloroplasts (22), but the stromal enzymes used in the present assay system are approximately 250 times more dilute than in the intact chloroplast. The increase in the concentration of the total fixed carbon pool per nmol CO_2 fixed, therefore, is correspondingly less in this system than in intact chloroplasts. When a relatively large amount of erythrose-4-P, in addition to priming amounts of 3-P-glycerate (Fig. 1B), was initially present in the assay, the rate of NADH oxidation and CO_2 fixation increased with time in a manner suggestive of autocatalysis, although a substrate amount of a cycle intermediate was present. The presence of a large pool of erythrose-4-P, by affecting the equilibrium of the transketolase reaction, would initially inhibit pentose-P formation from fructose-6-P and glyceraldehyde-3-P. This shows that in the absence of information on the concentrations of all relevant intermediates, care should be used in interpreting rate increases with time as being diagnostic of autocatalysis (23).

The demonstration here of oxidative PPC turnover and glycolytic activities in pea chloroplast extracts is consistent with the work of Stitt and ap Rees (21), who recently showed that starch breakdown and glucose metabolism by isolated intact pea chloroplasts involves oxidative PPC activity, with extensive recycling, as well as glycolytic activity. Kaiser and Bassham (7) previously reported that the oxidative PPC terminated with the formation of pentose-P in reaction mixtures based on spinach chloroplast extracts and containing 2 mM glucose-6-P. However, as acknowledged by these authors, the presence of high hexose-P/pentose-P ratios in their assay system may have prevented the thermodynamically unfavorable conversion of pentose-P to fructose-6-P.

In interpreting the results presented here, it has been assumed that the oxidative sugar phosphate metabolic activities observed were catalyzed by enzymes derived exclusively from the chloroplast stroma. The possibility of contamination of chloroplast extracts by significant amounts of enzymes of cytosolic origin has not been rigorously discounted. Such contamination is, however, unlikely since the chloroplasts were washed in a large volume of Medium 2 before the preparation of chloroplast extracts. Moreover, it has previously been established that chloroplasts from young pea leaves contain all enzymes necessary for catalysis of the complete oxidative PPC and of glycolysis as far as 3-P-glycerate (20).

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