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Author

Bassham, James A.

Publication Date

1977-02-01

Submitted to Encyclopedia of Plant
Physiology

LBL-6123
Preprint c1

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James A. Bassham

February 1977

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THE REDUCTIVE PENTOSE PHOSPHATE CYCLE
AND ITS REGULATION

The preparation of this paper was sponsored by the
U.S. Energy Research & Development Administration,
Division of Biomedical and Environmental Research.

Dr. James A. Bassham
Laboratory of Chemical Biodynamics
Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720

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A. Introduction

The reductive pentose phosphate cycle (RPP cycle) is the basic biochemical pathway whereby carbon dioxide is converted to sugar phosphates during the process of photosynthesis (16,17). This pathway is apparently ubiquitous in all photoautotrophic green plants (85), as it has been found to occur in all eucaryotic photosynthetic cells and in all blue-green algae so far examined. The RPP cycle also occurs in a variety of photosynthetic bacteria (112), although another cycle has been reported for some photosynthetic bacteria (49). In some higher plants, commonly designated C_4 plants, there is an additional pathway (C_4 cycle) via which carbon dioxide is first incorporated into four-carbon acids or amino acids and is later released to be refixed via the RPP Cycle (70,53,54).

The C_4 cycle is not an alternative to the RPP cycle. It does not result in any net reduction of CO_2 . Its function appears to be to utilize ATP from the photochemical reactions to bring carbon into the chloroplasts where the RPP cycle is operating. By this process, C_4 plants maintain a higher level of CO_2 at the local environment of the carboxylation enzyme of the RPP cycle, ribulose 1,5 carboxylase (RuDPCase). The result is to diminish the oxygenase activity of this enzyme (3,32,77) which can convert the carboxylation substrate, ribulose 1,5-diphosphate (RuDP) to glycolate, a substrate for the apparently wasteful process of photorespiration (129, 115,116). In addition, the C_4 pathway can recycle CO_2 that is produced within the cells by photorespiration (115).

The RPP cycle in eucaryotic cells occurs only inside the chloroplasts (5), the subcellular organelles which are also the site of the primary

photochemical reactions of photosynthesis in eucaryotic cells. The process of photosynthesis is complete within the chloroplasts, from the capture and conversion of light energy and the oxidation of water to molecular oxygen, to the uptake and reduction of carbon dioxide to starch and to triose phosphates which are exported from the chloroplasts to the cytoplasm (110,56,123,23,31).

The primary reactions of photosynthesis occur in the lamellae, or thylakoid membranes which contain the pigments, electron carriers, and other constituents involved in light absorption and conversion to chemical energy. The chemical energy derived from the primary reactions is used to drive electron transport and photophosphorylation (4). The overall result of electron transport in the membranes is the oxidation of water, giving O_2 , and the reduction of the non-heme iron protein, ferredoxin, to its reduced form (124). Photophosphorylation converts ADP and inorganic phosphate to ATP (5). The soluble cofactors, reduced ferredoxin and ATP, are the source of reducing equivalents and energy for the conversion of carbon dioxide to sugar phosphates in the chloroplasts.

The enzymes catalyzing steps of the RPP cycle are water soluble and are located in the stroma region of the chloroplasts (1). Only three steps in the cycle out of a total of thirteen actually use up cofactors (16,17) (ATP and NADPH) which must be regenerated at the expense of cofactors formed by the light reactions in the thylakoids. ATP is used directly, while NADPH is formed by reduction of $NADP^+$ at the expense of oxidation of two equivalents of ferredoxin:

There are no photochemical steps in the RPP cycle. There are, however, indirect effects of the light reactions on steps in the RPP cycle other than those requiring ATP or NADPH (87,22,14,72). These indirect effects on the catalytic activities of enzymes of the cycle are apparently mediated via changes in levels of cofactors and perhaps other electron carriers, and by changes in concentrations of certain ions, such as Mg^{+2} and H^{+} in the stroma regions of the chloroplasts where the soluble enzymes of the RPP cycle are located.

If the RPP cycle were the only metabolic pathway occurring in the stroma region inside the chloroplast envelope, and the only end product were starch, there might not be any need for metabolic regulation of the cycle. It would simply operate or not depending on the supply of NADPH and ATP from the light reactions and a supply of CO_2 . Following the mapping of the RPP cycle (16,17), not much effort was made to investigate its metabolic regulation for several years. It later became apparent that the metabolism of the chloroplasts is not limited to the RPP cycle, nor is reduced carbon withdrawn from the cycle at a single point for a single product. Metabolic regulation of the cycle was clearly needed at rate-limiting steps to allow for shifts in metabolism in the chloroplasts (for example, between light and dark)(14,22) and to keep the concentrations of intermediates of the RPP cycle within physiological ranges even as the demands on the cycle for various sugar phosphates as starting compounds for biosynthesis changed with the physiological needs of the plant (67,14).

Attention was focused on metabolic regulation of the RPP cycle by two kinds of experimental findings. One was the apparently anomalous transient changes in pool sizes of intermediate compounds in the chloroplasts observed when physiological conditions were suddenly changed. For example,

reactions catalyzed by enzymes not requiring cofactors from the light were seen to change in rate between light and dark (14,22,87). The other kind of experimental results leading to a study of regulation was the apparent inadequacy of the activities of some of the isolated enzymes catalyzing steps in the cycle (109,88). The key carboxylation enzyme, ribulose 1,5-diphosphate carboxylase (E.C. 4.1 1.39) appeared for years to have too high a K_m CO_2 value to accommodate the physiological levels of CO_2 normal for plants. The changes in enzyme activities, and the apparent inadequacies of the activities under suboptimal conditions have by now been mostly explained as resulting from the needs of metabolic regulation (12,13,14).

Information about the regulation of the RPP cycle has come from studies of the pool sizes of intermediate metabolites (often measured by using radioactively labelled substrates and measuring the labelling of the subsequently isolated metabolites)(72,24,22,66,87), and from measurement of O_2 evolution, phosphorylation, etc. in leaves, whole cells, isolated chloroplasts, and reconstituted chloroplasts (eg 80,81,119,120, 106,57,101,111). Much additional knowledge has come from studies of the individual enzymes, both in crude extracts and in isolated form. [Review, see Kelly, et al. (69).] Many of these investigations are reviewed in more detail in subsequent chapters of this volume (II. THE REDUCTIVE PENTOSE PHOSPHATE CYCLE). The present article will describe the RPP cycle, its mapping, the requirements for regulation, and the kinetic evidence, based on measurement of labelled metabolites in cells, chloroplasts, etc., for regulation in vivo. Some examples, as appropriate, will be given for the detailed mechanism of regulation of the enzymes, as determined by studies of the properties of the isolated enzymes. For more complete review of these properties of isolated enzymes, and for various other specialized aspects, the reader should refer to the later articles.

B. The Reductive Pentose Phosphate Cycle

1. The Cyclic Path of Carbon Dioxide Fixation and Reduction

Fixation and reduction of carbon dioxide via the RPP cycle follows a cyclic pathway (16,17). In the initial step of this cycle, RuDP is carboxylated and hydrolytically split to give two molecules of 3-phosphoglycerate (PGA). This C_3 acid then is phosphorylated and reduced to 3-glyceraldehyde phosphate (GA13P) via reactions using ATP and NADPH. The resulting triose phosphate then undergoes a series of isomerizations, condensations, and rearrangements which result in the conversion of five molecules of triose phosphate to three molecules of pentose phosphate, eventually in the form of ribulose-5-phosphate (Ru5P). This compound is then phosphorylated with ATP to regenerate the carboxylation substrate RuDP, thus completing the cycle.

A complete cycle, in which each reaction occurs at least once, is depicted in Fig. 1. The number of arrows represent the number of products and reactants participating in one complete cycle. Three molecules of RuDP are carboxylated to give six molecules of PGA, and after reduction, six molecules of GA13P. Only five of these GA13P molecules (15 carbon atoms) are required to regenerate the three RuDP molecules. The sixth GA13P molecule, equivalent in carbon to the three carbon dioxide molecules fixed, can either be converted by a reverse of glycolysis to glucose phosphate for starch synthesis, or can be exported from the chloroplast to the cytoplasm (23,110,56,123,9) for extrachloroplastic reactions. Other biosynthetic uses in the chloroplast are also possible; for example, conversion of GA13P to glycerol phosphate and eventually fats.

2. Individual Reactions of the RPP Cycle

a. The Carboxylation reaction.

In the initial step of the RPP cycle, the enzyme ribulose 1,5-diphosphate carboxylase (RuDPCase) catalyses the addition of CO_2 to C-2 of RuDP (121, 122, 93, 82). It is believed that an unstable enzyme-bound six-carbon intermediate molecule results, and that this intermediate is hydrolytically split with a concurrent transfer of a pair of electrons from C-3 of the RuDP to C-2. The detailed mechanism of this complex reaction is discussed later by Akazawa. The internal oxidation-reduction nature of this reaction led to the enzyme being called carboxydismutase for many years (41).

The forward reaction has a negative Gibbs free energy change of nearly 10 Kcal (24). This conversion of chemical energy to heat provides a substantial part of the driving force for the cycle and also facilitates the functioning of this enzyme in important regulatory roles, as will be discussed later. It also means that this reaction is essentially irreversible.

b. Reduction of PGA to GA13P

The reduction of PGA to GA13P occurs in two steps. First, the PGA is converted to the acyl phosphate in a reaction using ATP and mediated by PGA kinase (E.C. 2.7.2.3).

With equal concentrations or activities of reactants this reaction is highly unfavorable ($\Delta G' \approx 5$ Kcal) in the direction written, and it can only proceed as it does in photosynthesis by virtue of the relatively high physiological concentrations of PGA and such low concentrations of phosphoryl 3-phosphoglycerate, (PPGA) that the latter compound is not normally detected in experiments in vivo or with whole or reconstituted chloroplasts using $^{14}\text{CO}_2$ and ^{32}P -labelled phosphate.

The PPGA is reduced with NADPH and triose phosphate dehydrogenase (NADPH specific) (E.C. 5.3.1.1).

This reaction is also somewhat unfavorable energetically, but is helped by the fact that there are three products and only two reactants. The ratio $\text{NADPH}/\text{NADP}^+$ is probably not more than 3:1; (75) and the concentration of GA13P is not less than that of PPGA, but the production of P_i , when its concentration is about 1 mM, contributes -4.1 Kcal to the reaction (24).

The overall reaction, whereby PGA is phosphorylated and reduced to GA13P with ATP and NADPH, proceeds in the light under highly reversible conditions, and probably during photosynthesis is not subject to metabolic regulation. When the light is off, however, conversion of GA13P to PGA

may occur with a substantial negative ΔG^S , and it appears from reports described later that the oxidative sequence may well be regulated (2,86, 132,133,127,84,83,92,91) by mechanisms similar to those involved in other light-dark controlled steps. This sequence of reactions would thus be subject to light-dark regulation, but perhaps not to regulation during the period of active photosynthesis. This is in contrast to other regulated steps of the cycle which appear to be not only sites of light-dark regulation, but also to function as "fine-tuning" points of regulation in the light to keep in balance the concentrations of the various intermediate compounds of the RPP cycle (24).

c. Regeneration of Ru5P

A series of isomerizations and rearrangements are required for the conversion of five triose phosphate molecules to three pentose phosphate molecules. None of these reactions utilize light-generated cofactors (ATP and NADPH), and most steps are highly reversible. Two steps which liberate P_i are rate-limiting and have substantial negative ΔG^S values. Both are sites of regulation.

Two molecules of GA13P (per turn of the cycle) are converted with triose phosphate isomerase (E.C. 5.3.1.1) to dihydroxyacetone phosphate (DHAP).

In the presence of aldolase (E.C. 4.1.2.13), the two triose phosphates condense to give fructose 1,6-diphosphate (FDP) in a reversible step.

FDP is then converted to fructose 6-phosphate (F6P) with fructose diphosphatase (E.C. 3.1.3.11). This step has an estimated physiological $\Delta G^S = -7.0 \text{ Kcal (24)}$.

The conversion of triose phosphates and hexose phosphates to pentose phosphates, requiring changes in chain length, is initiated with transketolase (E.C. 2.2.1.1) which transfers C-1 and C-2 from F6P to GA13P, leaving the four carbon sugar phosphate, erythrose 4-phosphate (E4P), and forming xylulose 5-phosphate (Xu5P). This reaction is reversible.

The four-carbon aldose phosphate (E4P) can then condense with DHAP in a second reaction mediated by aldolase (E.C. 4.1.213) to give sedoheptulose 1,7-diphosphate (SDP). Like RuDP, this compound is unique to the RPP cycle and is not an intermediate compound in the oxidative pentose phosphate cycle (OPP cycle, also called the phosphogluconate pathway or the hexose monophosphate shunt).

This seven carbon ketose diphosphate is converted to sedoheptulose 7-phosphate and P_i with sedoheptulose 1,7-diphosphatase. It now appears that this enzyme is distinct from the fructose 1,6-diphosphatase. The reaction has a negative ΔG^S under physiological conditions of about -7 Kcal (24), and is a regulated and rate-limiting step.

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A second transketolase mediated step follows in which C-1 and C-2 of S7P are transferred to GA13P to give two pentose phosphates; Xu5P and ribose 5-phosphate (R5P). This completes the conversion of five GA13P molecules to three pentose phosphate molecules.

Before they can be used to regenerate RuDP, the Xu5P and R5P must be converted to Ru5P (E.C. 5.3.1.6 and 5.1.3.4).

d. Formation of RuDP

The final step in the RPP cycle is the conversion of Ru5P to RuDP with ATP and phosphoribulokinase (E.C. 2.7.1.19). This reaction has an estimated ΔG^S of about -4 Kcal, so that it is intermediate between those

reactions that are clearly reversible ($\Delta G^S = 0$ to -2 Kcal) and those that are almost completely irreversible ($\Delta G^S = -6$ to -10 or more Kcal). As discussed later and in subsequent articles there is much evidence that this step is metabolically regulated (2,127,128).

3. Stoichiometry and Energetics.

For each mole of CO_2 fixed by the cycle, one mole of ATP is required for the conversion of Ru5P to RuDP, the substrate for carboxylation. Two moles of ATP are required in the subsequent activation of the two moles of PGA formed, giving PPGA. The reduction of two moles of PPGA requires two moles of NADPH. For a complete turn of the RPP cycle, three moles of CO_2 are taken up, requiring the use of 9 moles of ATP and 6 moles of NADPH to make one mole of GA13P. This overall result can be expressed as the sum of two equations representing the utilization of the cofactors from the light, and another equation for the conversion of CO_2 , water, and P_i to GA13P.

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Subtracting the energy stored from the energy expended, we get $\Delta G' =$ only -33.9 Kcal as the driving energy for one turn of the cycle. The efficiency would be $350/384 = 91\%$. This calculation depends on the usual use of physiological free energy changes employed in biochemical energetics, but is somewhat misleading since actual physiological concentrations will always be much smaller than required to give activities of 1.0--in fact, they are generally in the range of 10^{-5} to 10^{-2} M. A more realistic estimate for the energetics can be made by using measured or estimated concentrations of metabolites as approximate values for the activities of reactants and products, and correcting the $\Delta G'$ values to physiological G^S values (24). This is particularly important with respect to the energy of hydrolysis of ATP to ADP and P_i , where $\Delta G'$ (with 10 mM Mg^{+2}) is about -7.6 Kcal, but ΔG^S is in the range of -12.5 to -13.5 Kcal, depending on ATP/ADP ratios and P_i concentrations.

When metabolite concentrations, measured and estimated in photosynthesizing Chlorella pyrenoidosa, were used in this way to estimate ΔG^S values (24), the energy input for 6 moles of NADPH and 9 moles of ATP is -427.0 Kcal, and the energy stored in making GAP from CO_2 , water, and P_i , with O_2 evolution is about + 353.6 Kcal. The chemical free energy converted to heat to drive the cycle becomes 73.4 Kcal, and the efficiency of the cycle is 83%. Of the 73.4 Kcal expended, about 30 Kcal are used in the carboxylation reaction, 12 Kcal in the phosphorylation of RuDP, and 19 Kcal in the FBPase and SDPase reactions. These four metabolically regulated steps account for 83% of the total energy expenditure, while the remaining 17%

is distributed among the remaining nine reactions, none of which are control points during photosynthesis (although some become rate limiting in the dark).

C. Utilization of the Products of the RPP Cycle

1. Starch Synthesis

Within the chloroplasts, the principle direct product of the cycle (in the absence of excessive photorespiration) is starch (51). The pathway to starch begins with conversion of GAl3P to F6P by reactions already discussed. There is no evidence to indicate that this conversion occurs at any different site or with different enzymes than those employed for the cycle. F6P is converted to glucose 6-phosphate (G6P) with hexose phosphate isomerase. Little or no free glucose is formed in photosynthesizing chloroplasts.

G6P is converted to G1P with phosphoglucomutase (E.C. 2.7.5.1). The reaction is somewhat "uphill" ($\Delta G' = +1.7$ Kcal) and is reversible, so that in vivo the ratio of G6P/G1P is about 20. The next step is the reaction of G1P with ATP, mediated by ADP glucose pyrophosphorylase and giving ADPglucose and inorganic pyrophosphate (PP_i).

As first shown by Ghosh and Preiss (52), the reaction catalyzed by ADP-glucose pyrophosphorylase is an important regulatory point. The enzyme activity is stimulated by PGA and is strongly inhibited by high levels of P_i . The concentrations of both can change and are believed responsible for starch regulation. Also, along with the rapid drop in ATP in the dark, decreased activity of the enzyme resulting from increased level of P_i

could account for the immediate drop in the level of ADPglucose when the light is turned off (12). Once formed, ADPglucose can transfer glucose to lengthen the anylose chain of a starch molecule.

2. Triose Phosphate Export.

The triose phosphates, GA13P and DHAP, were found to be the intermediate compounds of the chloroplasts that appeared to the largest extent in the medium of isolated spinach chloroplasts carrying out high rates of complete photosynthetic reduction of CO_2 (23). This and other studies (31, 110,123,56) suggest that these compounds are a form of photosynthetic product exported to the cytoplasm. A specific phosphate translocator apparently exists whereby the transport out of the chloroplast of triose phosphate is balanced by the movement into the chloroplast of inorganic phosphate (123,119,57). Once triose phosphate appears in the cytoplasm it can provide by its oxidation to PGA in the cytoplasm, both NADH and ATP (110,56). Besides the triose phosphates and P_i , it appears that PGA can move through the chloroplast envelope via the phosphate translocator mechanism. Two other compounds that were found among the intermediates rapidly appearing in the suspending medium of isolated photosynthesizing chloroplasts were FDP and SDP (23), but it now appears that these compounds were formed from triose phosphates by enzymes leaked into the medium from chloroplasts ruptured during the course of the experiments.

Due to this exchange, it can be seen that an increase in P_i in the cytoplasm could stimulate the export of triose phosphates from the chloroplasts via the phosphate translocator, with P_i entering and triose phosphates emerging (For Review, see Walker, 119). In fact, with isolated photosynthesizing spinach chloroplasts, an optimal level of P_i is needed for maximum

rates of CO_2 fixation. If there is too high a level of P_i , too many molecules of triose phosphate are lost from the chloroplasts, and the cycle becomes non-regenerative: Not enough RuDP is made to sustain high rates of carboxylation. On the other hand, some P_i is needed to sustain the RPP cycle which, especially in isolated chloroplasts, produces mostly PGA as the end product, and thus is using up P_i by forming organic phosphates. This presented a dilemma to research workers, who sought conditions for maintaining high rates of photosynthesis with isolated chloroplasts since no "phosphate-stat" was available, even if the problem had been recognized. Somewhat fortuitously, Jensen and Bassham (64) added inorganic pyrophosphate (PP_i) to the medium, and found substantial stimulation and prolongation of the CO_2 fixation rates. Apparently, inorganic pyrophosphatase (48) present in the medium converts PP_i to P_i , providing just the right amount if conditions are optimized (80,46). The pyrophosphatase activity requires Mg^{+2} , and too much of this ion causes P_i to be released too rapidly, leading to inhibition of CO_2 fixation due to too rapid translocation of triose phosphates out of the chloroplasts (80). Various combinations of Mg^{+2} and a fraction, first characterized by its fructose diphosphatase activity but containing pyrophosphatase, can cause very dramatic effects in increasing and decreasing the CO_2 fixation rates of isolated chloroplasts (18).

While it is not yet demonstrated that precisely such mechanisms are important in the in vivo regulation of photosynthetic metabolism, it seems entirely possible that such is the case. Many photosynthetic cells synthesize variable but substantial amounts of sucrose in the cytoplasm. This is true not only for higher plants, but also for the unicellular algae Chlorella pyrenoidosa (67). The formation of sucrose occurs via a mechanism similar to that in starch synthesis, but employing the reaction of G1P with UTP to produce UDPglucose which then reacts with F6P to form sucrose phosphate and

finally sucrose (73). The rate of PP_i formation in the UDPglucose pyrophosphorylase reaction is equivalent to the rate of sucrose formation, which in turn can often account for 10% or so of CO_2 fixation in Chlorella pyrenoidosa, and presumably could be a much higher proportion in mature leaves of higher plants that are exporting most of the photosynthetic product to other parts of the plant. The rate of conversion of PP_i to P_i must equal the rate of formation of PP_i by the sucrose synthesis and other biosynthetic reactions (such as protein synthesis) that produce PP_i . Nevertheless, the steady-state level of P_i could be sensitive to pyrophosphatase activity, which in turn may be regulated by such factors as Mg^{+2} level. This is because the total pool of inorganic phosphate is distributed between P_i , PP_i , and perhaps polyphosphates in Chlorella, so that the steady-state level of P_i depends on how much of the total phosphate is tied up in other forms. All this is somewhat hypothetical, but is discussed to illustrate one way that conditions in the cytoplasm, which is the immediate recipient of photosynthate from the chloroplast, might influence the RPP cycle occurring inside the chloroplast. The rate of withdrawal of triose phosphates affects the degree in which regulated rate-limiting steps of the RPP cycle must be balanced in order to maintain workable levels of cycle intermediates in the chloroplast.

3. Other Biosynthesis in the Chloroplasts.

Chloroplasts are capable of semi-autonomous growth and division within the cell, and it is clear that the chloroplasts have extensive biosynthetic capabilities for the formation of the enzymes of the RPP cycle and other needed soluble enzymes, and for all the components of the complex membrane system of the thylakoids, including pigments, cytochromes, phospholipids, galactolipids, membrane-bound proteins, etc. The fact that isolated photosynthesizing spinach chloroplasts do not appear to make substantial amounts

of all these substances from labelled CO_2 (e.g. 64) may mean that some intermediate steps must occur outside the chloroplasts, but it is not known which capabilities are missing from chloroplasts. The apparent absence of many enzymes of the tricarboxylic acid cycle suggest that conversion of PGA to keto acids, such as alpha ketoglutarate, may not occur in the chloroplasts.

In any event, it seems very likely that many of the carbon compounds needed for biosynthesis in chloroplasts are derived directly from intermediates of the RPP cycle. Examples are the synthesis of glycerol from DHAP and the diversion of R5P into ribonucleotide and deoxyribonucleotide synthesis. The magnitude of such diversions for biosynthesis is probably small compared to starch synthesis and the export of triose phosphates.

4. Glycolate Formation

a. Conditions Favoring Glycolate Formation

Under most conditions of photosynthesis in chloroplasts, some glycolate is formed (102,28). Under conditions which favor photorespiration, a very large part of the carbon fixed by the RPP cycle can be converted to glycolate (130,131). These conditions generally include high light intensity, low CO_2 pressure, more than a few percent O_2 , and temperatures above normal (20,126). Unicellular algae such as Chlorella pyrenoidosa which do not evolve photorespiratory CO_2 , form large amounts of glycolate under these conditions, and much of the glycolate is excreted into the medium. Even C_4 plants, which exhibit little or no photorespiration, form glycolate in amounts which increase with the conditions just listed. In the case of these plants, CO_2 formed by photorespiratory-type reactions is mostly recaptured in the leaves.

The two most widely proposed pathways by which glycolate can be formed in chloroplasts both involve oxidation of sugar phosphates that are inter-

mediate compounds of the RPP Cycle. There is considerable controversy over which pathway is more important, and there is evidence that both paths may operate, at least in Chlorella pyrenoidosa (21).

b. Formation from Sugar Monophosphates

Wilson and Calvin (126) observed greatly accelerated formation of glycolate in photosynthesizing algae when the levels of CO_2 in the gas bubbling through the algae were suddenly depleted. Since this was accompanied by an increase in levels of sugar monophosphates, they proposed that in the presence of transketolase the glycolyl moiety/normally transferred from a ketose monophosphate to an aldose phosphate acceptor instead is/oxidized to give glycolate. The dihydroxyethylthiamine pyrophosphate intermediate in the transketolase reaction can be converted to glycolate with ferricyanide (34). Shain and Gibbs (105) described a reconstituted preparation containing fragments of spinach chloroplasts, transketolase, and cofactors which is capable of rapid conversion of F6P or dihydroxyethylthiamine pyrophosphate to glycolate in the light.

c. Glycolate Formation From RuDP

Since phosphoglycolate is also seen upon the increase of O_2 concentration and decrease of CO_2 with photosynthesizing Chlorella pyrenoidosa, Bassham and Kirk (20) suggested that phosphoglycolate could be formed in vivo by the oxidation of RuDP.

Bowes, et al. (32,33) found that RuDP carboxylase also has an oxygenase activity that catalyzes the reaction producing phosphoglycolate. In the presence of $^{18}\text{O}_2$, this reaction produces glycolate with ^{18}O in the carboxyl group (77). Chloroplasts contain a specific phosphoglycolate phosphatase (95), which would convert phosphoglycolate to glycolate.

d. Which Glycolate Synthesis Pathway In Vivo?

Some evidence suggests that both pathways of glycolate synthesis may occur in photosynthesizing chloroplasts. Bassham and Kirk (21) performed a kinetic analysis of the appearance of $^{14}\text{CO}_2$ in RuDP, phosphoglycolate and glycolate before, during, and after the transition of photosynthesizing Chlorella pyrenoidosa from 1% $^{14}\text{CO}_2$ in air to pure O_2 . A temporary spike of phosphoglycolate and an elevated level of RuDP were accompanied by a nearly steady (slightly diminished after two min) generation of glycolate, most of which is not further metabolized in Chlorella. The simplest explanation of the kinetic data is that the activity of both RuDP carboxylase and RuDP oxygenase (the same protein) declined by two min after CO_2 removal, leading to a diminished rate of phosphoglycolate production. Such a decline in activity would perhaps be consistent with known regulatory properties of this enzyme discussed later.

Robinson and Gibbs (96) found that isolated spinach chloroplasts continue to produce glycolate even with saturating levels of CO_2 , when it is expected that the levels of RuDP would be very low. Eickenbusch et al. (47) showed that glycolate can be synthesized in a reconstituted chloroplast system from either F6P or RuDP. Only the synthesis from RuDP is sensitive to oxygen pressure.

It seems probable that the relative importance of the two pathways for glycolate synthesis varies with physiological conditions, and that it is perhaps premature, given the evidence available, to draw a firm conclusion

as to which pathway is predominant under the more common field conditions. The resolution of this problem is of considerable importance, since substantial efforts are being made to find ways to reduce photorespiration in C_3 plants (those plants with the RPP cycle but no C_4 cycle). One of the best ways to reduce photorespiration in such plants would seem to be to diminish glycolate formation from sugar phosphate intermediates of the RPP cycle, and it would be useful to know on which reaction to concentrate the research effort.

D. Mapping the RPP Cycle.

1. Early History

Once the basic equation of photosynthesis was established, with carbohydrates (CH_2O) considered to be the principal product (at least under conditions of high starch or sucrose synthesis), a simple formulation of the sequence of reactions of photosynthesis could be postulated. (For a complete discussion of the older theories of photosynthesis up to the 1940's, see Rabinowich, 94.) Early in this century it was believed that photochemical decomposition of CO_2 gave O_2 and some form of elemental carbon which could then undergo hydration to give carbohydrates. Some forms of this theory did not disappear until the 1950's.

In 1931, however, Van Niel (118) formulated photosynthesis as the transfer of hydrogen from water to carbon dioxide in algae and higher plants, and from other hydrogen donors to CO_2 in photosynthetic bacteria. The proposal that photosynthesis proceeds via the photochemical decomposition of water received further support when Hill and Scarisbrick (61) found that illuminated chloroplasts evolve O_2 when supplied with a suitable electron acceptor. In 1941, Ruben and coworkers (100) showed that O_2 evolved during photosynthesis agreed in isotopic composition with the oxygen

of the water rather than with that of CO_2 . With the discovery of short-lived radiocarbon (^{11}C) which could be used to label the CO_2 taken up by plants (99), Ruben and coworkers could prove that the fixation of CO_2 proceeds during the dark immediately following a period of illumination, and that the product of this fixation is an intermediate compound which can be subsequently transformed in the light (98). With the discovery of the long-lived radioisotope ^{14}C (97), the detailed pathway of photosynthetic carbon reduction could be mapped.

2. First Products of CO_2 Fixation.

From 1946 to 1953, Calvin and coworkers used ^{14}C as a radioactive label to follow the path of CO_2 fixation and reduction in photosynthesis (16,17,26, 27,28,^{29,}30,31,41⁴²). A few of the carboxylic acid products were identified following separation by solvent extractions and column chromatography (31) including ion exchange columns. The most useful separating and identification technique proved to be the resolution of the various acids and sugar phosphates by two dimensional paper chromatography and radioautography (29). The paper chromatographic methods had been developed for the analysis of amino acids by Martin and Singe (79). These methods plus radioautographic location of ^{14}C -labelled compounds on paper were applied to the analysis of ^{14}C -labelled amino acids formed during photosynthesis by Stepka, et al. (108) in 1948, and the solvent systems were then modified by Benson, et al. (29) to provide better separation of other products of CO_2 fixation.

The most heavily labelled compound after very short periods of photosynthesis with $^{14}\text{CO}_2$ was PGA (31). When this compound was chemically degraded, to permit measurement of the amount of ^{14}C in each carbon atom position, the predominant labelling at short times was in the carboxyl carbon (102). Such ^{14}C as was found in the remaining two carbon atoms

was at all times equally distributed between those positions. Such a finding immediately suggested that a cyclic process was involved, in such a way that the PGA was converted to a carboxylation substrate. By some process the label had become equally distributed between the two carbon atoms supplied by this substrate.

The plants used for most of the studies were either unicellular algae, Scenedesmus obliquus, or young leaves of higher plants such as barley or soybean, which we now know to be C_3 plants. Nonetheless the C_4 acids, malic acid and succinic, were among the early products of ^{14}C fixation, and these acids for a short time were thought to play a role in the cyclic regeneration of CO_2 acceptor. Possibly they could be split to give two C_2 pieces (31). It was soon found, however, that with malonate as an inhibitor, formation of these C_4 acids in algae could be suppressed without causing any decrease in either the photosynthetic uptake and reduction of CO_2 or in the appearance with time of ^{14}C label in the alpha and beta positions of PGA (15). The C_4 acids were thus dismissed as intermediate compounds in photosynthetic CO_2 fixation and reduction, and were not to reappear in such a role until the discovery of the special metabolism in certain "tropical grasses" such as sugar cane and maize, and in a scattering of other species (53,54,70).

3. Sugar Phosphates

Application of the two-dimensional paper chromatographic method to analysis of products of photosynthesis with $^{14}CO_2$ soon revealed that a number of sugar monophosphates and diphosphates were formed during the first few seconds of exposure to the labelled $^{14}CO_2$. Among these were DHAP, FDP, and G6P (29). Chemical degradation of the hexose phosphates showed the ^{14}C first appearing in the C-3 and C-4 positions, after which

the label appeared equally in C-1, C-2, and in smaller amounts in C-5 and C-6 (16). Such kinetics and pattern of labelling strongly suggested that the PGA, once formed, was converted to hexose monophosphates by a portion of the gluconeogenesis pathway from phosphoenolpyruvate (PEPA) to glucose. In turn, this proposition dictated that the cofactors required from the light reaction should be reduced pyridine nucleotides and ATP.

It was soon found that there were other sugar phosphates involved as early labelled intermediates. These were identified as SDP and S7P and as RuDP (28,26,40). Smaller amounts of Xu5P, Ru5P, and R5P were also found. Degradation of these compounds after short periods of photosynthesis with $^{14}\text{CO}_2$ revealed a pattern of labelling requiring the conversion of triose and hexose phosphates to pentose phosphates via some rearrangements of carbon chain length, as appears in the final version of the cycle (16,17).

The C-1 and C-2 atoms of hexose phosphates would be transferred to a triose phosphate giving pentose phosphate labelled at C-3 (derived from the carboxyl carbon of PGA via the aldehyde carbon of GA13P). The residual four carbon atoms of the hexose would then be E4P, labelled in C-1 and C-2 (derived from C-3 and C-4 of the hexose). The E4P, condensing with DHAP, also labelled in its terminal carbon atom (from the PGA carboxyl carbon) would give SDP and then S7P, predominantly labelled in C-3, C-4, and C-5. S7P and SDP were found to be so labelled. Transfer of the C-1 and C-2 of S7P to another molecule of GA13P would give R5P labelled in C-1, C-2, and C-3, and a molecule of Xu5P, labelled in C-3. When the two C-3 labelled Xu5P molecules and the C-1, 2, and 3-labelled R5P are converted to RuDP, the resulting molecule / ^{would have} label in the ratio 1:1:3 in positions C-1, C-2, and C-3. This was essentially the pattern found (16).

4. Studies of Light-Dark and High-Low CO₂ Transients

Further information about the sequence of events in the cycle came from studies of the changes in levels of labelled compounds accompanying sudden changes in physiological conditions (42,126). The unicellular algae, Scenedesmus obliquus, were allowed to photosynthesize with ¹⁴CO₂ in air for about 10 min. During this time samples of the algae were taken periodically, the algae in the sample were killed, and subsequently the labelled compounds were analyzed by two-dimensional paper chromatography and radioautography. After about five min photosynthesis with ¹⁴CO₂, the ¹⁴C content of intermediate compounds of the cycle no longer increases indicating that the compounds are fully labelled ("saturated"). After this time, the ¹⁴C content may be taken as a measure of the actual concentrations of the compounds in the actively turning over pools in the cells.

After saturation with ¹⁴C, the light was turned off, and more samples were taken in quick succession. Upon analysis, it was found that the concentration of PGA rose rapidly, indicating that cofactors generated by the light reaction are required for the subsequent conversion of PGA to sugar phosphates as expected (42). The rise in level of PGA for 20 sec or longer also indicates that the carboxylation reaction itself proceeds in the dark for a time, suggesting that cofactors from the light are not required for the carboxylation reaction.

After about 30 sec darkness, the PGA concentration falls. The carboxylation reaction by this time must have begun to deplete the supply of carboxylation substrate, whereas the dark reactions utilizing PGA exported from the chloroplast continue. Although, as discussed earlier, carbon export from the chloroplasts during photosynthesis is in the form of triose phosphates, when the light is turned off, PGA can be exported.

When the light is turned off, the concentrations of several sugar phosphates declined, but the most significant drop was in the level of RuDP, which in many experiments fell below detectable limits. When this was first observed by Calvin and Massini (42), they concluded that the step involved in the regeneration of RuDP requires a light-produced co-factor, namely ATP which is required for the conversion of Ru5P to RuDP with phosphoribulokinase.

In a similar experiment, Wilson and Calvin (126) first established steady state photosynthesis including ^{14}C -saturation with photosynthesizing Scenedesmus. Then they lowered the CO_2 level to nearly zero. In this case the carboxylation product, PGA, rapidly decreased in concentration, as expected, while the concentration of RuDP was the first among the sugar phosphates to rise. This provided direct in vivo evidence that RuDP is the carboxylation substrate in the RPP cycle. Since RuDP is a five-carbon compound, it was concluded that subsequent to the addition of CO_2 there must be a split to two three-carbon molecules. At least one of these products would have to be PGA, since PGA was shown to be the first product of CO_2 fixation. From a consideration of oxidation states of RuDP and CO_2 , both three-carbon products must in fact be PGA, if there is no external oxidant or reductant supplied to the reaction. Later studies with isolated RuDP carboxylase have provided overwhelming evidence for the addition of CO_2 to the C-2 of the RuDP, and an internal oxidation-reduction of the six-carbon intermediate, with hydrolytic splitting to give two molecules of PGA (82,93,121,122).

5. Discovery of Enzymes of the RPP Cycle

During approximately the period when the RPP cycle was mapped through the use of labelled carbon, an oxidative pentose phosphate cycle (OPP

cycle) was discovered by more classical biochemical methods in which the various enzymes required were isolated and characterized. Several of the reactions postulated for the OPP cycle appeared to be the reverse of reactions of the RPP cycle, and soon many of the required enzyme activities were isolated from green plants. For example, the trans-ketolase enzyme, essential for both cycles, was purified from spinach by Horecker and coworkers in 1953 (62). Pentose phosphate isomerase was found in alfalfa (6). The finding of these and other enzyme activities of the OPP cycle and glycolysis provided much of the necessary supporting biochemical evidence for the RPP cycle.

There are, however, three enzyme activities unique to RPP cycle. Of these, perhaps the most important in establishing the cycle is the RuDPCarboxylase. The enzymic carboxylation of RuDP in vitro was first reported by Quayle and coworkers (93) who demonstrated, in 1954, the formation of PGA, labelled with ^{14}C in the carboxyl group only, when RuDP and $^{14}\text{CO}_2$ were added to a cell-free extract obtained from Chlorella. The enzyme was purified and characterized by Weissbach and coworkers (121, 122) soon afterwards, and there has been a large amount of work on the properties of this enzyme since then (reviewed by Akazawa, this volume).

A second key enzyme unique to the RPP cycle is phosphoribulokinase, purified from spinach by Hurwitz and coworkers in 1956 (63). The third unique enzyme is sedoheptulose 1,7-disphosphatase (SDPase). For a long time it was thought that this enzyme might be identical to fructose 1,6-diphosphatase, but recent work by Buchanan et al. (38) shows it to be derived from FDPase when FDPase is converted from its dimer to its monomer form.

E. Metabolic Regulation of the RPP Cycle

1. In Vivo Kinetic Steady-State Studies with Labelled Substrates

The methods of kinetic analysis of measuring levels of labelled metabolites (see D. 4.) have also proved useful in the identification of sites of metabolic regulation. The steady-state levels of radioactive intermediate compounds can be used to calculate the physiological free energy changes/for a specified plant and set of physiological conditions. (ΔG^S) This information provides a direct measure of the reversibility of the reactions as they are occurring in vivo (24). It can be easily shown that the relation between ΔG^S and the reversibility of the reaction is given by:

$$\Delta G^S = -RT \ln(f/b)$$

where f is the forward reaction rate and b the back reaction rate.

In order for such measurements to be meaningful, accurate procedures for the maintenance of steady-state conditions and continuous measurement of CO_2 , specific radioactivity, rapid sampling and killing, and quantitative analysis of radioactivity in each compound as a function of the amount of tissue sampled were developed (19). Initially, the steady-state kinetic method was used to demonstrate amino acid formation directly from photosynthate without the intermediacy of sucrose or starch in photosynthesizing Chlorella pyrenoidosa (107). Some years later, the method was used to determine the ΔG^S values for reactions of the RPP cycle, as already discussed in B. 2. and as summarized in Table 1 (24). The reactions shown to be rate-limiting in the light (during photosynthesis) were those mediated by RuDPCase, FDPase, SDPase, and phosphoribulokinase. As it happened, these were four of the five reactions for which there was already in vivo evidence for light-dark regulation. The three reactions with the highest negative free energy

changes were catalyzed by enzymes which in their isolated state had been reported to have insufficient catalytic activity to accommodate the requirements of the RPP cycle (109,88).

2. Light-Dark Regulation

a. Respiratory Metabolism in Photosynthetic Cells and Chloroplasts

When the light is turned off with Chlorella pyrenoidosa which were previously photosynthesizing under steady-state conditions, a number of interesting transient changes in the pool sizes of intermediate compounds occur in addition to the changes in PGA and RuDP described in D. 4. Further changes were revealed by using ^{32}P -labelled phosphate in addition to $^{14}\text{CO}_2$, and by turning the light on again after allowing enough time to establish a new steady-state condition of respiratory metabolism in the dark (22,12-14,87). There is an immediate appearance of labelled 6-phosphogluconate in the dark, and an equally rapid disappearance in the light. This intermediate is unique to the OPP cycle.

With whole cells, the operation of the OPP cycle, indicated by the appearance of 6-phosphogluconate, could be occurring in the chloroplasts, the cytoplasm, or both. In fact, Heber, et al. (55) found that the unique enzymes of the OPP cycle, glucose phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, are present in both cytoplasm and chloroplasts of spinach and Elodea, with the larger amounts located in the cytoplasm.

Other studies of photosynthesis in isolated spinach chloroplasts in the presence of Vitamin K_5 , which is thought to divert electrons from the light reaction and thus mimic aspects of dark metabolism, demonstrated the formation of 6-phosphogluconate in chloroplasts (72).

When both ^{14}C and ^{32}P labelled substrates were administered to photosynthesizing Chlorella and the light was turned off following over 11 min photosynthesis with $^{14}\text{CO}_2$, the curves diverged for the two isotopes in a given compound such as 6-phosphogluconate or any of the sugar phosphates, with the ^{32}P labelling becoming greater compared with ^{14}C labelling with time in the dark (87,22,66). While intermediate pools were saturated with both labels in the light, and ^{32}P labelling continues to be saturated in the dark, it was clear that dark respiration utilized endogenous compounds that were not fully ^{14}C -labelled during the period of photosynthesis with $^{14}\text{CO}_2$. It is noteworthy that the level of ATP in the Chlorella cells, while dipping about 40% during the first minute in the dark, was reestablished at the same steady state level as in the light after about 10 minutes of darkness.

Studies of the disappearance of labelled starch and sucrose in Chlorella in the dark following a long period of photosynthesis with $^{14}\text{CO}_2$ were particularly revealing with respect to the source of respiratory carbon in chloroplasts and cytoplasm, in the absence or presence of intracellular ammonium ion and as substrates for the oxidative pentose phosphate cycle (66). The preliminary period of photosynthesis and the first part of the respiratory period were without added NH_4^+ in the suspending medium. When the light was turned off, sucrose synthesis stopped, but there was no decline in the level of labelled sucrose; hence, it was concluded that sucrose was not contributing to the respiratory metabolism.

In contrast, the level of labelled starch immediately began to decline, and continued to decline for the duration of the experiment at a very substantial but constant rate. Immediately after the light was turned off, the level of 6-phosphogluconate rose, and other changes in the sugar phosphate levels were indicative of operation of the OPP cycle. Since starch in

Chlorella is in the chloroplasts, this OPP cycle activity probably occurs inside the chloroplasts. It might be argued that the starch was converted to triose phosphate and PGA via glycolysis, since phosphofructokinase activity has been found in chloroplasts (68). The conversion via the OPP cycle seems more probable in the Chlorella experiment since the level of FDP (as well as of SDP) dropped precipitously during the first minute of darkness, and then returned to higher levels in the dark than in the light over a five minute period, whereas the levels of pentose phosphates remained nearly constant.

When the light was turned off, labelled sucrose remained constant, and only began a steady decline (at about half the rate of starch utilization) when 1 mM NH_4^+ was added to the suspending medium (66). Coincident with this change was a second rise in 6-phosphogluconate, and again temporary declines in the levels of FDP and SDP, followed by increases to new high levels. These and other changes suggested that the utilization of sucrose, located in the cytoplasm, was unaffected by darkness but dependent on intracellular NH_4^+ , with utilization occurring via the OPP cycle in the cytoplasm (66).

Given the larger ratio of chloroplast volume to cell volume in Chlorella as compared to cells of leaves, it is perhaps to be expected that the OPP cycle/may be quantitatively less important in leaf cells than in the unicellular algae, particularly in view of the findings that the larger amounts of the OPP cycle enzymes are in the cytoplasm in leaf cells (55). Nevertheless, it appears likely that the OPP cycle does operate as an important respiratory pathway in leaf chloroplasts, and similar regulatory mechanisms are probably required in all chloroplasts of eucaryotic cells to ensure an efficient switch from photosynthetic to respiratory metabolism.

b. Light-Dark Regulation of FDPase and SDPase

Early examination of the activities of enzyme extracts from leaves showed limiting and possibly inadequate catalysis of the conversion of FDP and SDP to the respective monophosphates (88,109). It was therefore of interest when light-dark and dark-light transient changes in levels of these metabolites in Chlorella, previously labelled with ^{14}C and ^{32}P during photosynthesis, showed unexpected kinetics (22,87). When the light was turned off, the levels of both of these compounds dropped rapidly, as would be expected with the cessation of the reduction of PGA to triose phosphates. Then, over a period of ten minutes, the levels of these compounds rose again, a greater increase in ^{32}P labelling than in ^{14}C labelling. As discussed earlier, this indicates the respiratory breakdown of endogenous carbohydrates that were only partially labelled in the light.

When the light was turned on again, there was a very rapid buildup in the levels of FDP and SDP (as well as DHAP) for about 30 sec, with the levels reaching higher than steady-state light levels. Then there was an equally rapid drop in these levels for another 30 sec, followed by damped oscillations leading to a steady-state light level equal to that achieved in the previous light period.

The interpretation of these interesting kinetics is that when light is turned on, there is a rapid reduction of PGA to triosephosphates which are rapidly converted to FDP and SDP. The "overshoot" in the levels is attributed to the diphosphatases having become inactive in the dark period, and requiring about 30 sec in the light to become reactivated. During this period, the level of F6P and S7P also drop--further indication that the diphosphatases are inactive. With this and other blocks in the RPP cycle, regeneration of RuDP is limited, carboxylation does not reach steady-state rates, and the rate of formation of PGA and of its conversion to triose

phosphates and FDP and SDP is limited. After 30 seconds, when the diphosphatase become fully active, levels of these diphosphates fall sharply, as these compounds are converted to sugar monophosphates and eventually to RuDP. With removal of the diphosphatase blocks and other blocks, the RPP cycle reached full velocity, and soon the rate of reduction of PGA to triose phosphates and consequent formation of FDP and SDP is sufficient to bring the levels of these compounds to their steady state light values. Although such a control mechanism results in a surging of carbon around the RPP cycle, the oscillations are quickly damped (only about two are seen) due to the metabolite pools acting as capacitors and the rate limiting steps acting as resistors.

The inactivation of the FDPase and SDPase activities in the dark are required to prevent the operation of futile cycles in the dark, when respiratory metabolism occurs in the chloroplasts. The buildup in the levels of FDP and SDP seen in the dark may be due to operation of the OPP cycle, of glycolytic conversion of G6P to FDP via F6P and phosphofructokinase, or both. As is well known in non-photosynthetic cells, phosphofructokinase and FDPase activity, when both present in the same cellular compartment, must be so regulated that they are not both active at the same time, lest a futile cycle operate to hydrolyze ATP.

In the case of SDPase activity in the dark, a somewhat more extended futile cycle could operate, in which S7P and GA13P would be converted to F6P and E4P/ (which should be present if there is an OPP cycle operating). This E4P plus a molecule of DHAP then would condense to give SDP. The F6P with ATP and phosphofructokinase would give FDP and hence GA13P and DHAP, completing the futile cycle. Similar futile cycles would occur in the light, (as is likely) unless/any phosphofructokinase activity present in the chloroplasts is inactivated in the light.

The prevention of such futile cycles requires that, in some cases, enzyme activities be capable of more variation than can be achieved with a single regulatory effector or inhibitor. FDPase and SDPase, like several other enzymes of the RPP cycle and the OPP cycle in chloroplasts, seem to be regulated by more than one factor that changes in concentration between light and dark. In common with some other regulated chloroplast enzymes, FDPase and SDPase respond to changes in pH and Mg^{+2} . Increased Mg^{+2} lowers the pH optima of these enzymes (90,50). Since both Mg^{+2} (76,11,10,60,71) and pH (102,59) increase in the light in chloroplasts, the combined change has a substantial effect on enzyme activity.

A second major regulation of SDPase and FDPase depends on another important change between light and dark: the level of reduced ferredoxin. Recently, two small protein factors, ARP_a and ARP_b have been described (38,39,127,128,103,104) which are involved in the activation of FDPase and SDPase in the presence of reduced ferredoxin (35,36,37). One of the factors, ARP_b , has proved to be identical with thioredoxin, an enzyme factor found in many cells besides photosynthetic cells, and involved in the conversion of ribonucleotides to deoxyribonucleotides. The other factor ARP_a functions as ferredoxin-thioredoxin reductase. In the presence of optimal levels of Mg^{+2} and at optimal pH, there is still considerable activation of FDPase and SDPase by added ARP_a and reduced ferredoxin.

These results appear to be compatible with reports of inhibitor studies by Anderson and Avron (2) which implicated a factor at the level of ferredoxin in the activation of the diphosphatases.

c. Light-Dark Regulation of RuDP Carboxylase

When Chlorella pyrenoidosa are allowed to photosynthesize with $^{14}CO_2$ under steady-state conditions with a total CO_2 pressure comparable to that

in air (0.03%), the steady-state level of RuDP is quite high: over 0.4 mM in the cells as a whole, and probably more than 2 mM in the stroma region of the chloroplasts (24). When the light is turned off, the level of RuDP declines rapidly for the first two minutes and then reaches a level about 0.05 of the light level from which it declines very slowly. Since the K_m for RuDP for the fully activated enzyme is about .035 mM (45), and the $\Delta G'$ for the carboxylation reaction is -8.4 Kcal (24), this failure for the reaction to continue after two minutes of darkness means that the enzyme activity has greatly declined.

The light-dark inactivation of the RuDPCase is also evident with isolated spinach chloroplasts (22) where, following a period of photosynthesis with $^{14}\text{CO}_2$, the level of the RuDP in the dark declined to about one half the light value and then remained constant. When the light was again turned on, the level of RuDP rose very rapidly for 30 sec and then declined to the light level. This behavior is analogous to that of the changes in FDP and SDP levels described above and attributed to dark inactivation of diphosphatase activity, followed by light reactivation requiring 30 sec.

When the drop in RuDP level in the isolated spinach chloroplasts was prevented by addition of ATP to the suspending medium just after the light was turned off, very little uptake of $^{14}\text{CO}_2$ occurred as long as the light was off (even though there was as much RuDP present in the chloroplasts as in the light). When the light was turned on again, high rates of $^{14}\text{CO}_2$ uptake resumed (65). Although the rate of entry of ATP into whole chloroplasts may be low compared to the requirements of photosynthesis (56,58,111) this low rate is apparently sufficient to maintain the level of RuDP when it is not being consumed, once the RuDP carboxylase is inactivated.

Like the activities of FDPase and SDPase, RuDP carboxylase activity depends in part on pH, Mg^{+2} and reduced cofactors, but the mechanisms are

different. The control of the carboxylase activity is complicated by the necessity for the plants to avoid, at least to some extent, the wasteful conversion of RuDP by oxygenase activity to phosphoglycolate and PGA (3,32, 77). Molecular oxygen, O_2 , can bind competitively at the CO_2 binding site of the carboxylase. This O_2 binding is thus favored by high O_2 and low CO_2 pressures. Low CO_2 pressures in the light can cause an increased level of RuDP, providing optimal conditions for the oxygenase reaction. To avoid this reaction, it is advantageous for the enzyme to be inactivated with respect to O_2 binding by a combination of high concentration of RuDP and low concentration of CO_2 . Apparently, the binding of O_2 can only be decreased by a change in conformation of the enzyme which results in increased binding constants for both CO_2 and O_2 .

Isolated RuDP carboxylase is activated by preincubation with CO_2 or bicarbonate and high levels of Mg^{+2} (e.g. 10 mM), before the enzyme is exposed to RuDP (89,43,44,45). Preincubation with physiological levels of RuDP in the absence of either bicarbonate or Mg^{+2} results in conversion of the enzyme to an inactive form with high K_m values for CO_2 , and the enzyme does not recover its activity for many minutes upon subsequent exposure to physiological levels of bicarbonate and Mg^{+2} (43,44). Full activation of the isolated purified enzyme requires that the preincubation with CO_2 and Mg^{+2} also be carried out in the presence of either NADPH or 0.05 mM 6-phosphogluconate, each at physiological levels (44,45, 43).

With respect to light-dark regulation, it seems clear that the changes in Mg^{+2} levels and pH in the chloroplasts which affect FDPase and SDPase activities, also result in changes in RuDPcarboxylase activity, with the light-induced increases in pH and Mg^{+2} resulting in increased enzyme activity. The pH optimum of the isolated enzyme shifts towards the pH actually found in chloroplasts in the light (about 8) with increased Mg^{+2} ,

and the value of K_m for CO_2 is lower at pH 8 than at pH 7.2 (113,78,25).

The activation of the isolated enzyme by NADPH seems to be another part of the light-dark regulation, but the activation by 6-phosphogluconate is at first surprising, since this compound appears in the dark. Kinetic studies show that the 6-phosphogluconate is still present during the first two minutes of light after a dark period (22), and it may be that a useful activation occurs then, while the level of NADPH is still being built up (43). In the dark, 6-phosphogluconate would not activate the carboxylase since the optimal conditions of pH and Mg^{+2} levels would not be met.

Presumably, any light activation of the enzyme via NADPH would involve the transfer of electrons to NADP^+ via ferredoxin. This does not explain how oxidized ferredoxin could activate the isolated RuDP carboxylase, as reported by Vaklinova and Popova (117), and confirmed in the author's laboratory (unpublished work).

Although it appeared for many years that KmCO_2 for RuDP carboxylase is too low to support the RPP cycle, a number of laboratories have found evidence in recent years that the KmCO_2 is sufficiently low. In particular, Bahr and Jensen (8) found that a low KmCO_2 form of the enzyme obtained from freshly lysed spinach chloroplasts could be stabilized with dithioerythritol, ATP, MgCl_2 , and R5P. Moreover, with optimal conditions of pH and Mg^{+2} ions, and in the presence of physiological levels of NADPH in the light, the apparent $K_m\text{CO}_2$ values of the carboxylase in reconstituted chloroplasts are well below the level of CO_2 in air (0.035%)(unpublished work, this laboratory). McC. Lilley and Walker (81) have shown that the activity and KmCO_2 for the enzyme from spinach chloroplasts are more than adequate to support photosynthesis.

d. Light-dark Regulation of Phosphoribulokinase

In vivo light-dark transient studies are not so revealing with respect to regulation of the conversion of Ru5P to RuDP since the level of the substrate ATP declines rapidly in the dark. In vivo evidence for the inactivation of the enzyme came from studies in which Vitamin K₅ was added to photosynthesizing Chlorella pyrenoidosa (72). The result was that electrons were diverted from the reduction of ferredoxin to the reduction of the oxidized form of Vitamin K₅, but there was little effect on the level of ATP. Consequently, those reactions needing reduced cofactors in the chloroplast were affected, but not via ATP levels.

Upon the addition of Vitamin K₅ to the algae, there was an immediate increase in 6-phosphogluconate and in pentose monophosphates, but a rapid drop in the level of RuDP. It appears that the OPP cycle became activated, but that the conversion of Ru5P to RuDP ceased. This is in agreement with the known properties of the isolated enzyme. The same protein factor, ARP_b, implicated in FDPase and SDPase activation causes activation of phosphoribulokinase in the presence of dithiothreitol (104). Since ARP_a, ferredoxin-thioredoxin (ARP_b) reductase is present in the chloroplasts, it is reasonable to expect that the same activation mechanism may operate for phosphoribulokinase as for FDPase and SDPase. The inhibitor study of Anderson and Avron (2), however, suggested that a different membrane-bound reducing factor might be involved in the activation of phosphoribulokinase, and this point remains to be clarified.

e. Light-Dark Regulation of Glyceraldehyde Phosphate Dehydrogenase

Since the reduction of phosphoryl 3-phosphoglycerate (PPGA) is the second step in the conversion of PGA to GA13P, and the two steps require ATP and NADPH, light-dark transition studies reveal little about the possible regulation of the reactions. From steady-state kinetic tracer studies of photosynthesis of Chlorella pyrenoidosa it seems clear that neither step is

rate limiting in the light (24), and it thus appears doubtful that regulation in the light is required. There is however, considerable enzymatic evidence that the enzymes catalyzing the oxidation of triose phosphates are subject to regulation (2,84,91,92,83,127,132,133).

In the light, during photosynthesis, the concentrations of PGA, NADPH, ATP and GA13P are such that the overall reaction from PGA to GA13P is highly reversible. In the dark, however, the drop in levels of both NADPH and ATP in the chloroplasts (75) results in the oxidation reaction being highly favored, and limitation on the rate of oxidation may be required in order to maintain levels of GA13P sufficient for operation of the OPP cycle in the chloroplasts.

f. Regulation of Glucose-6-Phosphate Dehydrogenase

Prevention of the OPP cycle in the chloroplasts during photosynthesis requires that the glucose-6-phosphate dehydrogenase be inactivated. The sudden appearance of 6-phosphogluconate in the dark and its disappearance in the light have already been mentioned. Not surprisingly, glucose-6-phosphate dehydrogenase is inactivated with increasing ratios of NADPH/NADP⁺ (74,125), and with changes in this ratio equal to those actually observed in chloroplasts between light and dark, there is a large change in the activity of this enzyme. The activity is further affected by RuDP and by pH in the directions expected to inactivate in the light (74).

3. Regulation of the RPP cycle During Photosynthesis

Besides the "On-Off" kind of regulation required for transition from photosynthesis to respiration, a finer tuning of rate-limiting steps is required to keep in balance the concentrations of intermediate compounds as the physiological needs and rates of photosynthesis of the cells change (67). The possibilities for factors in the cytoplasm to influence the relative amounts of triose phosphates exported from the chloroplasts (see C.2) mean

that the rates of formation and conversion of GA13P via the cycle must be adjustable. The rate-limiting steps in the light are the formation and the carboxylation of RuDP and the conversions of FDP and SDP to F6P and S7P respectively. Thus the rate of the carboxylation reaction relative to the diphosphatase reaction determines the rates of formation and utilization of triose phosphates. The reduction of PGA to triose phosphates and the conversion of triose phosphates to FDP and SDP are highly reversible, and therefore play no role in controlling triose phosphate concentrations during active, unimpaired photosynthesis.

Probably there is much more to be learned about the way in which this is accomplished, but one possible mechanism is in the sigmoidal dependence of FDPase activity on FDP concentration⁽⁹⁰⁾. If the GA13P level were to drop too much due to triose phosphate export, then the levels of DHAP and of FDP would also drop, the FDPase activity would decline, and triose phosphate concentration would build up. Undoubtedly, other mechanisms are required as well for the levels of triose phosphates and also for the levels of hexose, heptose and pentose monophosphates.

F. Concluding Remarks

Since the mapping of the RPP cycle 25 years ago, there has accumulated a wealth of information about the control of the cycle, the enzymes of the cycle and their regulation, the nature of export of photosynthate from the chloroplasts, and the physical and chemical variations in the chloroplasts with light, dark, and changing physiological conditions. The origin of glycolate, the substrate for photorespiration, remains in dispute, but much has been learned about glycolate formation by oxidation of sugar monophosphates and of RuDP. The C_4 cycle of certain plants was discovered, and

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we now know much about the way plants with this cycle minimize the adverse effects of photorespiration. Many of these topics have been mentioned very briefly in this article, but the chapters that follow will no doubt describe fully the progress in each. It is with this knowledge that the author has neglected much important work and concentrated to a great extent on work from his own laboratory in order to provide one perspective.

TABLE I

Free Energy Changes of the RPP Cycle

The standard physiological Gibbs free energy changes (G') were calculated for units activities, except $[H^+] = 10^{-7}$. The physiological free energy-changes at steady-state are for a 1% w/v suspension of *Chlorella pyrenoidosa* photosynthesizing with 0.04% $^{14}CO_2$ in air and with other conditions as described by Bassham and Krause (). The stroma concentrations were assumed to be four times the total cellular concentrations, and are used as approximations for activities.

Reaction	$\Delta G'$	ΔG^S
$CO_2 + RuDP^{4-} + H_2O \longrightarrow 2 PGA^{3-} + 2 H$	-8.4	-9.8
$H^+ + PGA^{3-} + ATP^{4-} + NADPH \longrightarrow ADP^{3-} + Ga13P^{2-} + NADP^+ + P_i^{2-}$	+4.3	-1.6
$Ga13P^{2-} \longrightarrow DHAP^{2-}$	-1.8	-0.2
$Ga13P^{2-} + DHAP^{2-} \longrightarrow FDP^4$	-5.2	-0.4
$FDP^{4-} + H_2O \longrightarrow F6P^{2-} + P_i^{2-}$	-3.4	-6.5
$F6P^{2-} + Ga13P^{2-} \longrightarrow E4P^{2-} + Xu5P^{2-}$	+1.5	-0.9
$E4P^{2-} + DHAP^{2-} \longrightarrow SDP^{4-}$	-5.6	-0.2
$SDP^{4-} + H_2O \longrightarrow S7P^{2-} + P_i^{2-}$	-3.4	-7.1
$S7P^{2-} + Ga13P^{2-} \longrightarrow R5P^{2-} + Xu5P^{2-}$	+0.1	-1.4
$R5P^{2-} \longrightarrow Ru5P^{2-}$	+0.5	-0.1
$Xu5P^{2-} \longrightarrow Ru5P^{2-}$	+0.2	-0.1
$Ru5P^{2-} + ATP^{4-} \longrightarrow RuDP^{4-} + ADP^{3-} + H^+$	-5.2	-3.8
$F6P^{2-} \longrightarrow G6P^{2-}$	-0.5	-0.3

Figure 1.

The Reductive Pentose Phosphate Cycle

The heavy lines are for reactions of the RPP cycle, the faint lines indicate removal of intermediate compounds of the cycle for biosynthesis. The number of heavy lines in each arrow equals the number of times that in the cycle occurs for one complete turn of the cycle, in which three molecules of CO_2 are converted to one molecule of GA13P.

Abbreviations: RuDP, ribulose 1,5-diphosphate; PGA, 3-phosphoglycerate; DPGA, 1,3 diphosphoglycerate; GA13P, 3-phosphoglyceraldehyde; DHAP, dihydroxyacetone phosphate; FDP, fructose 1,6-diphosphate; F6P, fructose 6-phosphate; G6P, glucose 6-phosphate; E4P, erythrose 4-phosphate; SDP, sedoheptulose 1, 7-diphosphate; S7P, sedoheptulose 7-phosphate; Xu5P, xylulose 5-phosphate; R5P, ribose 5-phosphate; Ru5P, ribulose 5-phosphate; TPP, thiamine pyrophosphate.

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This report was done with support from the United States Energy Research and Development Administration. Any conclusions or opinions expressed in this report represent solely those of the author(s) and not necessarily those of The Regents of the University of California, the Lawrence Berkeley Laboratory or the United States Energy Research and Development Administration.

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LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720