

Lawrence Berkeley National Laboratory

Recent Work

Title

THE MECHANISMS OF THE REACTIONS OF THE CARBON REDUCTION CYCLE OF PHOTOSYNTHESIS

Permalink

<https://escholarship.org/uc/item/1pg9w9s0>

Author

Bassham, J.A.

Publication Date

1962-08-01

University of California

Ernest O. Lawrence
Radiation Laboratory

TWO-WEEK LOAN COPY

*This is a Library Circulating Copy
which may be borrowed for two weeks.
For a personal retention copy, call
Tech. Info. Division, Ext. 5545*

Berkeley, California

DISCLAIMER

This document was prepared as an account of work sponsored by the United States Government. While this document is believed to contain correct information, neither the United States Government nor any agency thereof, nor the Regents of the University of California, nor any of their employees, makes any warranty, express or implied, or assumes any legal responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by its trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof, or the Regents of the University of California. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof or the Regents of the University of California.

Lecture - American Society of Plant Physiologists
Corvallis, Oregon, August 1962

UCRL-10521

UNIVERSITY OF CALIFORNIA

Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

THE MECHANISMS OF THE REACTIONS
OF THE CARBON REDUCTION CYCLE
OF PHOTOSYNTHESIS

J. A. Bassham

August 1962

The Mechanisms of the Reactions of the Carbon Reduction Cycle
of Photosynthesis

J.A. Bassham

The basic carbon reduction cycle of photosynthesis, mapped by Calvin and coworkers^{1,2,3} consists essentially of four stages. First, ribulose phosphate is phosphorylated with ATP from the light reactions of photosynthesis to give ribulose-1,5-diphosphate. Second, ribulose diphosphate is carboxylated and subsequently gives two molecules of 3-phosphoglyceric acid. Third, 3-phosphoglyceric acid is reduced with ATP and TPNH from the light reactions to give triose phosphate. Fourth, a series of condensations, chain length dismutations and rearrangements converts five molecules of triose phosphate to three molecules of ribulose-5-phosphate.

Bassham and Kirk⁴ showed that in Chlorella this basic cycle appears to account for at least 70% of the total carbon fixation during photosynthesis. Their kinetic data suggested that the carboxylation reaction does not give rise to two molecules of phosphoglyceric acid in isotopic equilibrium with the free pool of 3-phosphoglyceric acid. This data might be interpreted as supporting the proposal of Wilson and Calvin⁵ that the carboxylation reaction in the light in vivo might be reductive, thus producing one molecule of triose phosphate and one molecule of 3-phosphoglyceric acid, containing the newly incorporated carbon dioxide.

Enzyme activities catalyzing all the stages in the carbon reduction cycle have been isolated from green tissues^{3,6}. Studies of the enzymic activities by Peterosky and Racker⁷ indicated somewhat inadequate activities for the carboxylation enzyme carboxydismutase, and low activities for fructose diphosphate-1-phosphatase and sedoheptulose diphosphate-1-phosphatase. Richter⁸ and Fewson et al.^{9,10} found that certain organisms lacked fructose diphosphate aldolase, the enzyme postulated for the formation of fructose diphosphate from triose phosphates.

These apparent enzymic deficiencies have suggested to some¹¹ that the postulated carbon reduction cycle is incorrect. However, the preponderance of kinetic data from tracer studies³ strongly supports a carbon reduction cycle identical with or very similar to the one which has been proposed. It is very difficult to conceive of another metabolic pathway which could account for all these data without calling on reactions which are thermodynamically unfavorable and for which no enzymic precedent exists such as the direct reduction of carbon dioxide to some one or two carbon compound¹¹.

Perhaps it is incorrect to assume that the carbon reduction cycle of photosynthesis is mediated by a collection of individual, separate enzymes in contact with only free pools of cycle intermediates all in solution in some subcellular compartment. The concept of organized, multifunctional enzyme systems, capable of performing a sequence of biochemical steps on bound intermediates has received a tremendous support from the work of Lynen¹². He has reported strong

evidence that fatty acid synthesis takes place via a series of seven steps, the first of which is the transfer of the malonyl moiety from malonyl coenzyme A to the sulfhydryl group of an enzyme. During subsequent steps this malonyl moiety is condensed with acyl CoA and the product is not released from the enzyme until it has been reduced to a saturated fatty acid. The bound substrate moiety is pictured as being acted upon sequentially by first one enzyme function and then another.

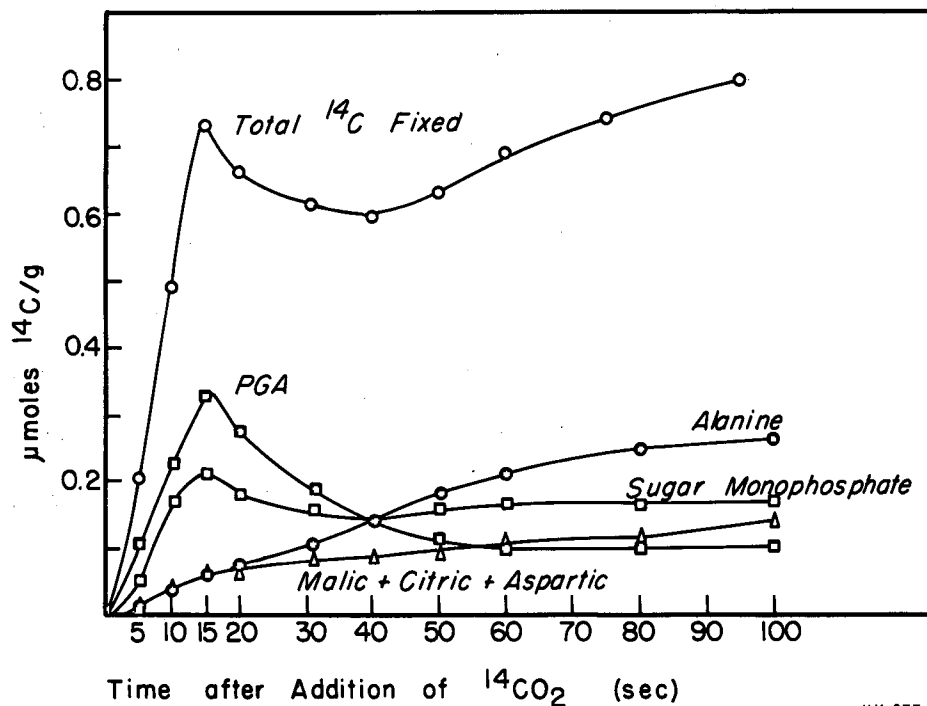
The kinetic and thermodynamic advantages of such a multifunctional organized enzyme system for a biosynthetic pathway are important. More efficient utilization of enzyme functions, prevention of excessive energy loss due to hydrolysis of chemically active functional groups such as acid anhydrides, and avoidance of competitive inhibition by naturally occurring intermediates are some of these advantages.

In view of the known efficiency and rapidity of the reactions of carbon reduction during photosynthesis, one is led to suspect the existence of a multifunctional organized enzyme system for this process. Its basic importance as the primary biosynthetic reaction and its long evolutionary history would seem to require that carbon reduction in photosynthesis be mediated by the most efficient system possible in living cells.

Arguments based upon kinetic studies with carbon 14, and upon direct enzymic evidence will be presented here in support of the thesis that the reduction of carbon in photosynthesis via the carbon reduction cycle is mediated by such an organized, multifunctional enzyme system. I shall propose a detailed biochemical mechanism for the operation of this system.

In studies of the kinetics of carbon 14 dioxide reduction in Scenedesmus during photosynthesis², it was found that upon extrapolation to zero time of the percentage of the total fixation into specific compounds, the percentage in phosphoglyceric acid approached about 75%. Some of the other radiocarbon at very short time was found in malic acid, presumed to be a product of a noncyclic C₁-C₃ carboxylation. However, extrapolation to zero time showed about 18% of the total radioactivity incorporated into certain sugar phosphates, particularly fructose and sedoheptulose monophosphates and diphosphates. (Benson et al.¹³ had already showed that the activity in glucose and mannose monophosphate extrapolates to zero at zero time.) Thus, it could be concluded that certain sugar phosphates which are intermediates in the carbon cycle might be formed from enzyme-bound phosphoglyceric acid which in turn is formed by the primary carbon fixing reaction.

Recently, Bassham and Kirk¹⁴ investigated the formation of ¹⁴C-labeled compounds by Chlorella when ¹⁴CO₂ was administered to the Chlorella in the dark immediately following a period of illumination and photosynthesis with unlabeled radiocarbon (Fig. 1). During the first 15 sec of exposure of the plants to radiocarbon in the dark, the rate of incorporation of ¹⁴C into sugar phosphates appeared to be too rapid to be accounted for by reduction of labeled PGA in the free pool of PGA. Thus, it was again concluded that some bound form of the carboxylation product was reduced directly on the enzyme.



MU-27743

Fig. 1. Fixation of ^{14}C into compounds when $^{14}\text{CO}_2$ is administered to previously photosynthesizing *Chlorella* in the dark. (See ref. 14.) The $^{14}\text{CO}_2$ was administered to the $2\frac{1}{2}\%$ suspension (wet packed/volume/suspension volume) in the steady-state apparatus⁴ within one sec after the lights had been turned off placing the algae in darkness. Samples were taken every few sec as shown, were killed in 80% methanol in water (final composition) at room temperature, and were concentrated in vacuo and analyzed by two-dimensional chromatography and radioautography. Radioactivity of compounds was determined by counting them, on the chromatograms, with a G. M. tube and scaler.

In the same studies, it was noted that once the ^{14}C label had been incorporated in these sugar monophosphates in the dark, it was only partly lost from them by reoxidation during a subsequent dark period between 15 and 40 sec., when presumably all the reducing power that had been generated in the light was exhausted. After 60 sec no further loss of label from sugar phosphates occurred. In the same experiment, there was a slow but continuous incorporation of radiocarbon into aspartic acid, in the dark following the initial 60 sec period. This suggested a continuous $\text{C}_1\text{-C}_3$ carboxylation followed by transamination exchange of the presumed oxalacetic acid product. On the other hand, malic acid, though labeled during the first 30 sec, was subsequently not further labeled. Malic acid is supposedly formed by reduction of $\text{C}_1\text{-C}_3$ carboxylation product by TPNH. If sugar phosphates were formed during photosynthesis by reduction of phosphoglyceric acid with TPNH, then one might expect that in the dark the same sugar phosphates would be oxidized by TPN^+ to give TPNH, which could then be used to reduce the $\text{C}_1\text{-C}_3$ carboxylation product thus producing more malic acid.

The failure of the sugar phosphates to be oxidized in the dark and the cessation of labeling of malic acid, taken together with the continued labeling of aspartic acid, suggest strongly that there is some enzymic block to the oxidation of sugar phosphates by TPN^+ . This sort of block to oxidative reactions is probably important and necessary to the operation of the reductive carbon cycle of photosynthesis. It is one of the keys to the mechanism of the enzyme system which will be proposed.

Another important key is the distribution of radiocarbon in the

central carbon atoms of hexose and heptose monophosphates isolated from plants following short periods of photosynthesis in the presence of $^{14}\text{CO}_2$. Kandler and Gibbs¹⁵ found that under certain conditions carbon 4 is significantly more labeled than carbon atom 3 in hexose monophosphates. Bassham et al.² found that carbon atom 4 in sedoheptulose monophosphate is considerably less labeled than carbon atoms 3 or 5. In fact, the radiocarbon in carbon atom 4 seemed to extrapolate to zero at zero time of exposure of soy bean leaves to $^{14}\text{CO}_2$.

In the cycle as usually formulated, carbons 3 and 4 of hexose monophosphate give rise to carbons 4 and 5 respectively, of sedoheptulose monophosphate. Therefore, the two experimental observations are related. Both suggest that hexoses are formed by condensation of two kinds of triose phosphate molecules which are not in isotopic equilibrium with each other. Moreover, the less labeled of these two triose phosphates should be dihydroxyacetone phosphate since its terminal carbon atom would be the precursor for carbon atom 3 of hexose and carbon atom 4 of heptose.

A bound form of phosphoglyceraldehyde which could give rise to a free form of phosphodihydroxyacetone (in an essentially irreversible reaction) could account for such a labeling pattern, provided the bound phosphoglyceraldehyde could be formed by reduction of the newly incorporated $^{14}\text{CO}_2$. The necessity, based on kinetic data, for a reduction of a bound form of phosphoglyceric acid has already been indicated. Such a reduction would lead directly to a bound form of phosphoglyceraldehyde.

By far the most likely candidate for a binding function for acids and aldehydes is an enzyme sulfhydryl group. For reasons which will be discussed later, I suggest that the binding site for acids and aldehydes in the carbon reduction cycle is an enzyme disulfide capable of reduction in the light to enzyme disulfhydryl. In its reduced form, this site is presumed to be a reducing agent of strength comparable to San Pietro's^{16,17} PPNR, which has been found by Tagawa and Arnon¹⁸ to be as good a reducing agent as H₂.

In any proposal of a multifunctional enzyme system for the carbon reduction cycle of photosynthesis, one must also provide for a function which includes bound thiamine pyrophosphate (TPP), for TPP is necessary for the transketolase reactions of the carbon reduction cycle^{19,20,21,22,23,24}. Upon examining various known mechanisms of the carboxylation reactions, I became convinced that the most plausible type of mechanism for the carboxylation of ribulose diphosphate is analogous to a reverse of the already well-established mechanism for the decarboxylation of pyruvic acid^{23,25,26,27}. In this latter mechanism, pyruvic acid reacts with TPP by addition of the carbonyl carbon to the carbon atom of the thiazole ring between the nitrogen and sulfur atoms, after which decarboxylation gives an acetaldehyde TPP compound. This compound reacts with oxidized lipoic acid to give acetyl hydrolipoic acid and TPP.

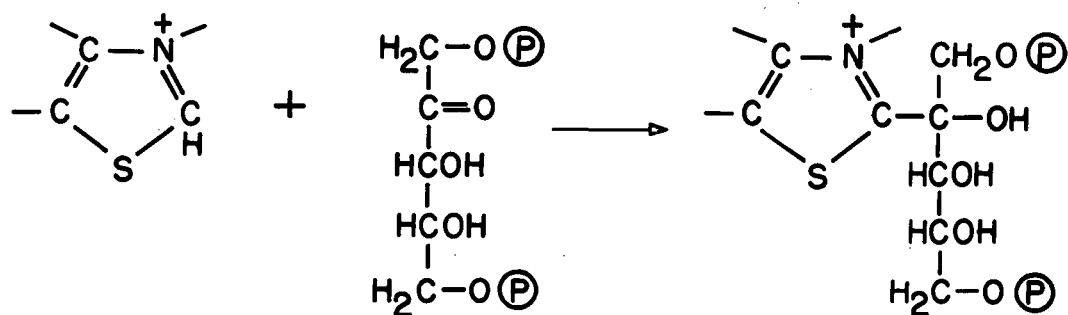
Using these two functional groups, disulfhydryl and thiazole ring, I shall now present a scheme for the mechanism of the carbon reduction cycle. With it before us, the subsequent kinetic and enzymic evidence can be discussed more easily.

Suppose that ribulose-1,5-diphosphate could add to TPP bound to the enzyme. (The thiamine should be such a bound form, for addition of TPP to isolated carboxydismutase does not stimulate its activity - it may in fact inhibit.) There would result a ribulose diphosphate-TPP addition compound. (Fig. 2) In a transketolase type reaction, a phosphoglyceraldehyde molecule would be released, but in the case of ribulose there is a different configuration of hydrogen and hydroxyl at the number 3 carbon atom (compared with fructose and sedoheptulose). Besides, the phosphoglyceraldehyde must be kept bound for the subsequent in vitro reaction which is about to be described.

Let us suppose that the thiazole ring opens and that the phosphoglyceraldehyde moiety transfers electrons to the thiazole carbon by the rupture of the C-S bond of the thiazole ring and simultaneous formation of a phosphoglyceryl-S bond. (For details see Fig. 3.) Now the glycolaldehyde carbon bonded to the thiazole carbon can carry a partial negative charge, and carboxylation can take place, somewhat analogous to a reversal of the decarboxylation of pyruvic acid. Thus carboxyphosphoglycolaldehyde TPP would be formed (Fig. 4).

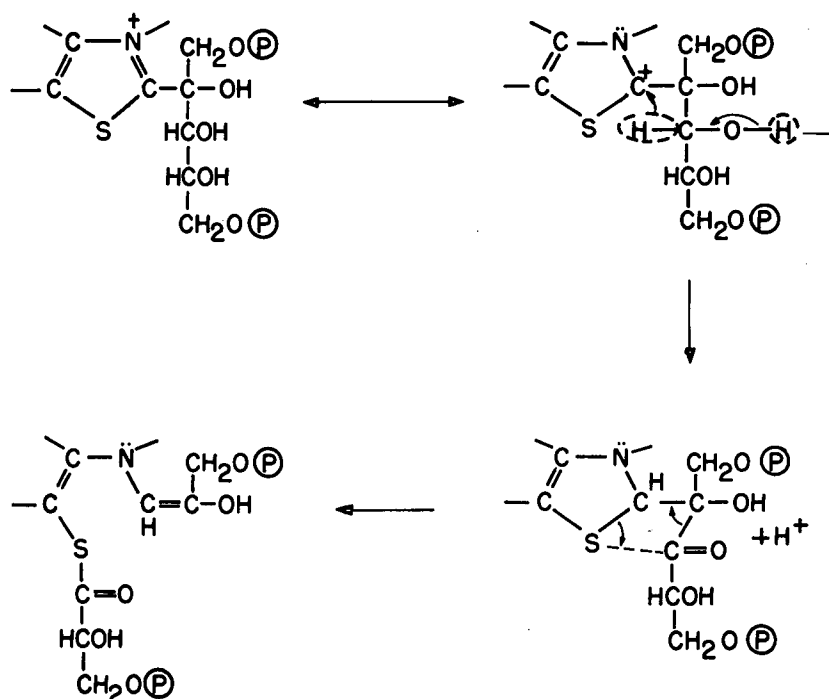
In vitro, or in the dark, with no strong reducing enzyme functional group present, hydrolysis of the phosphoglycerylsulfur bond (OH^- attack) and of the thiamine carboxyglycolaldehyde moiety bond (H^+) with ring closure to reform the thiazole ring would result in the effective transfer of electrons to the carboxyglycolaldehyde moiety and the formation of two molecules of 3-phosphoglyceric acid (Fig. 5).

In vivo in the light, if there were present^a/strong reducing group, such as enzyme disulfhydryl, with a redox potential of hydrogen (-0.42 v) it could accept the phosphoglyceryl moiety from the TPP sulfur (forming sulfhydryl) and hold it as 3-phosphoglyceryl-S-enzyme-SH.



MU-27715

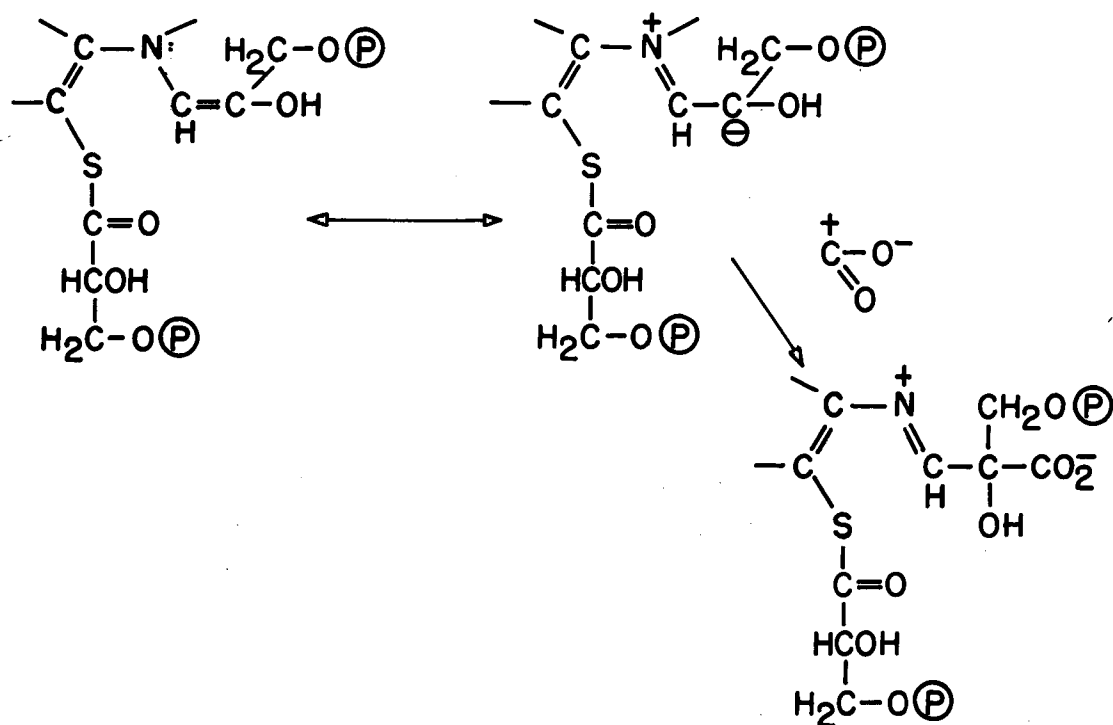
Fig. 2. Proposed mechanism of the carbon reduction cycle. Reaction of TPP with ribulose diphosphate. The carbonyl carbon of ribulose-1, 5-diphosphate adds to the acidic number 2 position of the thiazole ring of TPP in a typical condensation, analogous to the addition of fructose-6-phosphate to TPP in the transketolase reaction.



MU-27716

Fig. 3. Proposed mechanism of carbon reduction cycle. Rearrangement of ribulose diphosphate-TPP addition compound.

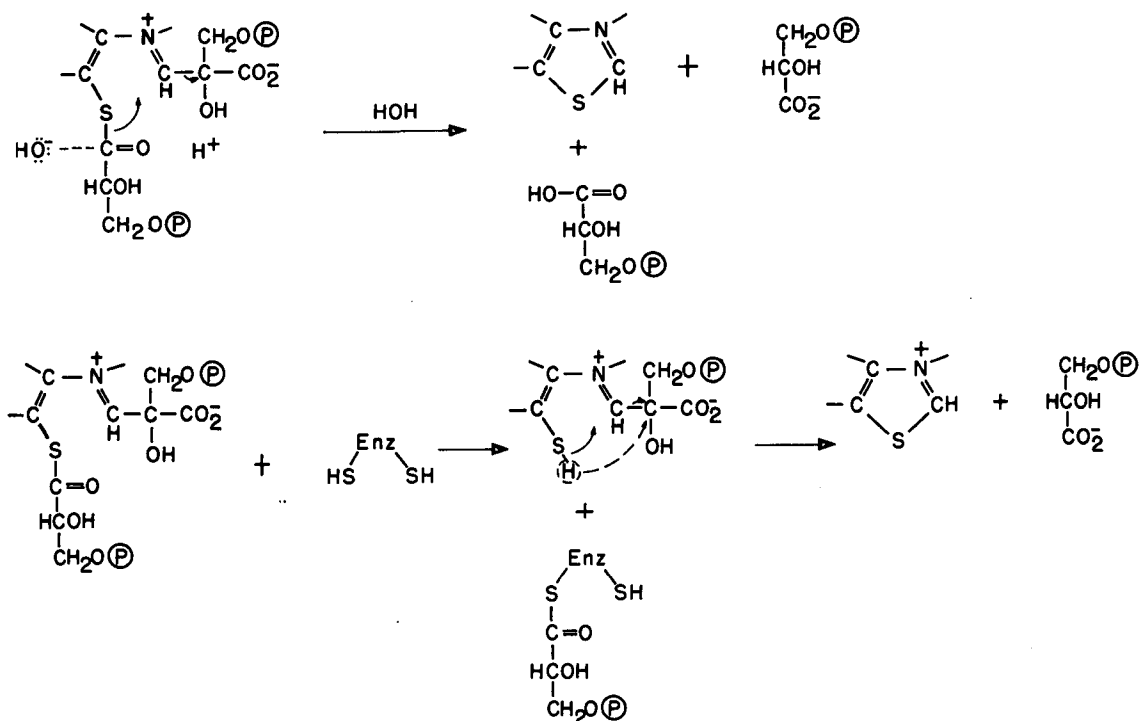
In the addition compound, the number 2 carbon atom of the sugar is asymmetric. Since the reaction is enzyme mediated, the product will be one optical isomer, the sugar moiety being oriented on the enzyme by the configuration of its asymmetric carbons. The configuration of the carbon atoms 2 and 3 of the transketolase type addition compounds (such as are formed with xylulose-5-phosphate) are not known, but must be opposite from the configuration of the hydroxyl groups on carbons 2 and 3 of ribulose diphosphate addition compound. Because of this difference, one can visualize different reaction courses in the two cases. In the case shown here hydride ion from sugar carbon atom 3 migrates to the thiazole carbon 2 and the hydroxyl loses a proton so that a carbonyl group is formed. The resulting carbonyl carbon attracts the sulfur atoms and electrons, opening the thiazole ring and breaking the C-C bond of the sugar as shown.



MU-27717

Fig. 4. Proposed mechanism of the carbon reduction cycle:
Carboxylation reaction.

Once the thiazole ring has been opened, and the phosphoglyceryl moiety transferred to the sulfur atom, the resonance hybrid shown in which the number 2 carbon atom of the sugar has an electron excess can exist. Carboxylation then occurs with the CO₂ molecule. The resonance form of the CO₂ with an electron deficient carbon atom is probably stabilized by an enzyme function and Mg⁺⁺ ion, perhaps as Enz-Mg-O- $\overset{+}{\text{C}}\begin{matrix} \text{O} \\ \parallel \\ \text{O} \end{matrix}$. The reaction shown is analogous to the reverse of the decarboxylation of pyruvate.



MU-27718

Fig. 5. Proposed mechanism of the carbon reduction cycle: A. Hydrolysis of the carboxylation product, B. Partial reduction of the carboxylation product.

A. In vitro and in vivo in the dark there would be no disulfhydryl reducing agent. Hydrolysis, initiated by hydroxide ion attack on the acyl sulfur bond causes release of 3-phosphoglyceric acid and thiazole ring closure. Transfer of electrons to the carboxyglycol-aldehyde moiety allows it to be released as a second molecule of 3-PGA.

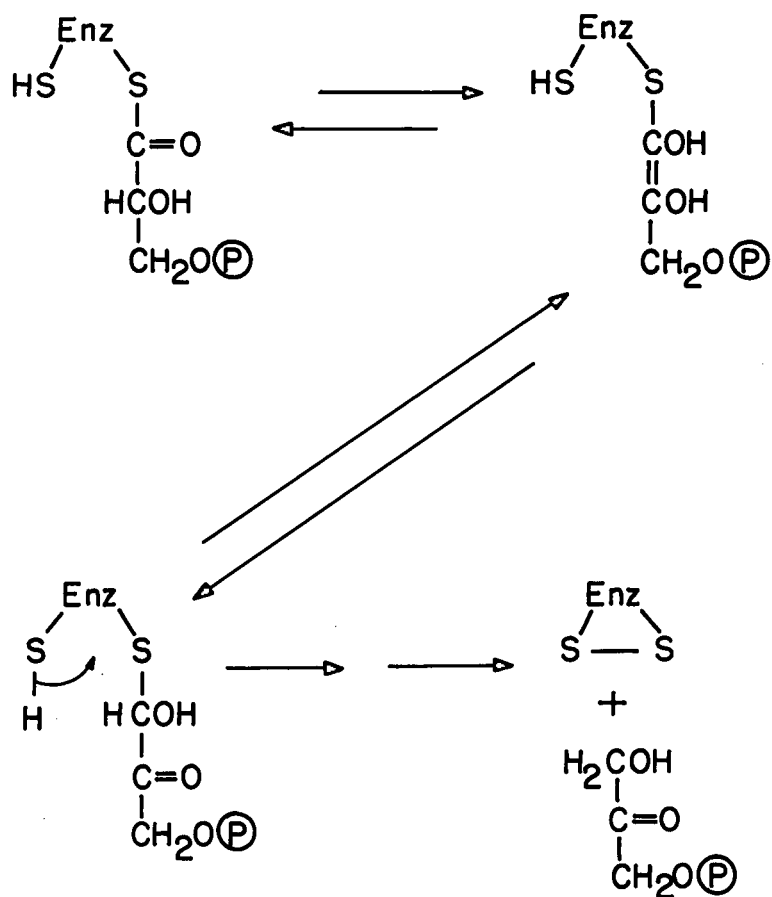
B. In vivo in the light disulfhydryl enzyme is present to accept the 3-phosphoglyceryl moiety, and to convert the thiazole sulfur to sulfhydryl. The ring closes, and electrons are then transferred to the carboxyglycolaldehyde which is released as 3-PGA. The trouble with this mechanism is that the latter molecule of 3-PGA would contain all the newly incorporated carbon, whereas kinetic data (see text) indicate this PGA should be reduced while still attached to the enzyme.

Rearrangement and internal oxidation of a molecule of this enzyme-bound three-carbon moiety gives dihydroxyacetone phosphate and oxidized enzyme disulfide (Fig. 6). Another molecule of the phosphoglyceryl-S-enzyme-SH can react with free phosphodihydroxyacetone to give enzyme-disulfide and fructose-1,6-diphosphate (Fig. 7).

The carboxyphosphoglycolaldehyde thiamine pyrophosphatesulfhydryl would, if hydrolyzed, give TPP and free 3-phosphoglyceric acid. As already mentioned, some phosphoglyceric acid should be reduced without coming free from the enzyme.

Such a reduction requires phosphorylation of the carboxyl group followed by reduction with a suitable reduced cofactor. The phosphorylation might be accomplished by ATP. Since a TPP had been reported in plants²⁸, this high energy phosphate anhydride could very well be the immediate phosphorylating agent (Fig. 8).

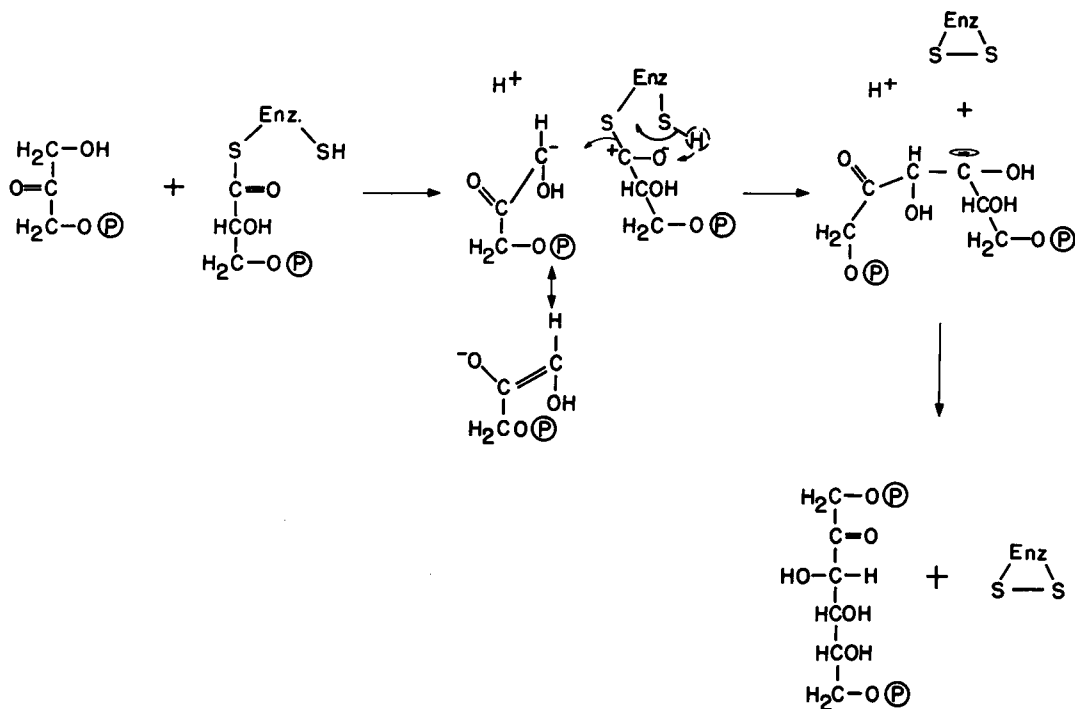
Once the acyl phosphate were formed, the thiazole sulfhydryl could bond to the acyl carbon, releasing inorganic phosphate and forming a seven membered ring (Fig. 9). This compound would then react with another enzyme disulfhydryl, giving back the TPP and phosphoglyceryl-S-enzyme-SH, identical with the one already formed. Hydrolysis of this compound would give only phosphoglyceric acid. In vivo, some molecules of this compound would be converted to phosphodihydroxyacetone (DHAP) and others would react with DHAP to give fructose diphosphate as mentioned above. At very short exposure of plants to $^{14}\text{CO}_2$, radioactive carbon in the DHAP pool would be diluted, and the resulting fructose would be more labeled in carbon atom 4 than in carbon atom 3 (Fig. 10).



MU-28185

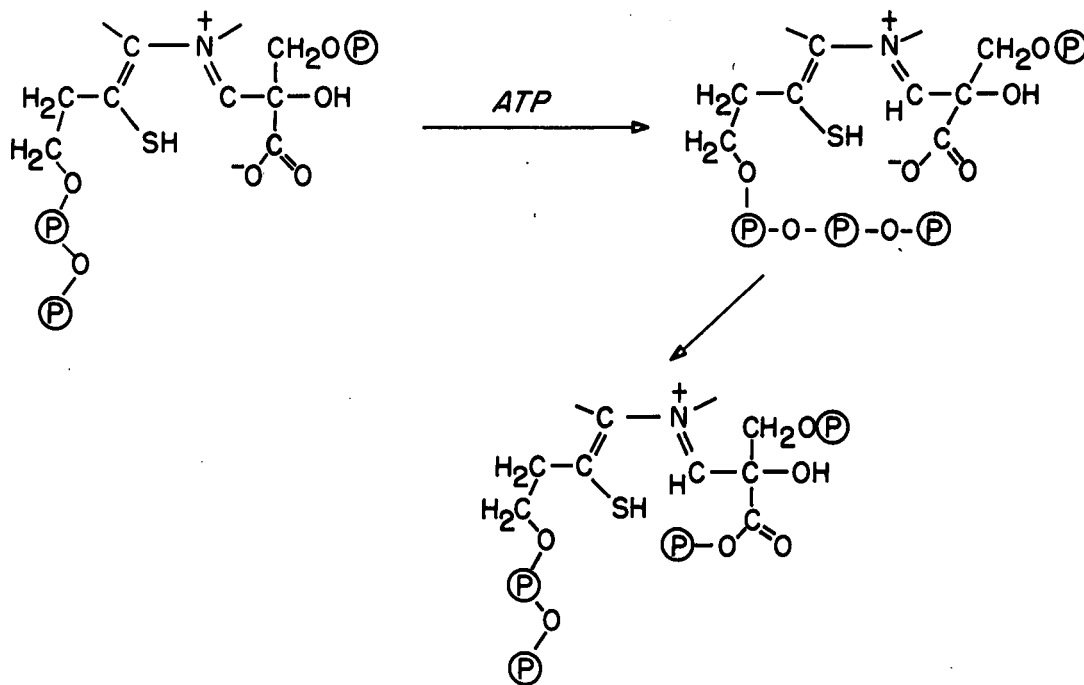
Fig. 6. Proposed mechanism of the carbon reduction cycle. Formation of dihydroxyacetone phosphate.

By keto-enol shifts, hydrogen atoms are transferred to carbon atom 1 of the 3-phosphoglycerate moiety. The resulting weakening of the S-C bond permits the disulfide bond formation with accompanying reduction and release of the three carbon compound as DHAP.



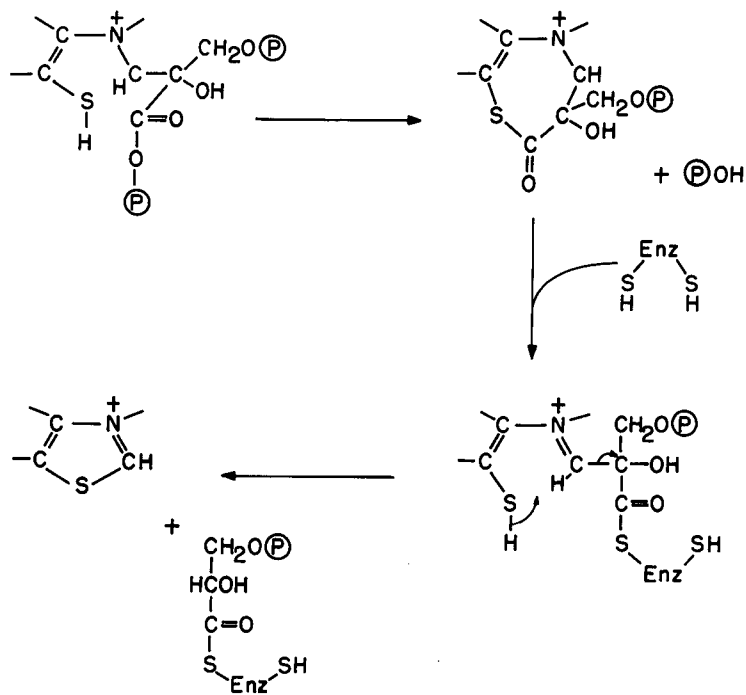
MU-27719

Fig. 7. Proposed mechanism of the carbon reduction cycle. Condensation of phosphoglyceryl-S-enzyme-SH with dihydroxyacetone phosphate to give fructose-1,6-diphosphate. The aldehyde hydrogen of usual aldol type condensation is here replaced by -S-enzyme-SH which supplies electrons for the new C-C bond, as well as a proton directly to the carbonyl oxygen, and the energy released by forming the disulfide bond.



MU-28186

Fig. 8. Proposed mechanism of the carbon reduction cycle. Phosphorylation of carboxyl group. With ATP, the carboxyglycolaldehyde thiamine triphosphate addition compound is formed. The terminal phosphate is transferred to the carboxyl group, forming an anhydride, which will be more reactive for the subsequent reduction. (See Fig. 9).

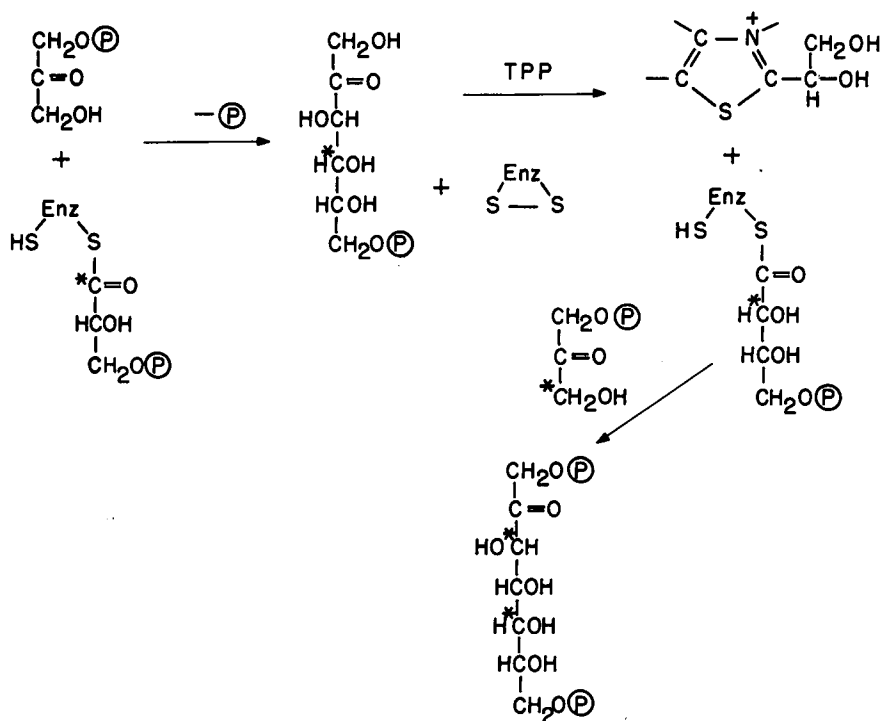


MU-28187

Fig. 9. Proposed mechanism of the carbon reduction cycle.

Reduction of acyl phosphate.

The sulfhydryl group of the thiamine reduces the acyl phosphate, releasing inorganic phosphate and forming a seven membered ring. The C-S bond is then transferred to the reduced enzyme disulphydryl. Finally, the thiazole ring closes, releasing the phosphoglyceryl-S-enzyme-SH.



MU-27744

Fig. 10. Proposed mechanism of the carbon reduction cycle. Formation of fructose and sedoheptulose diphosphates and explanation of labeling data. Condensation of phosphoglycerate, labeled after very short exposures of the plant to $^{14}\text{CO}_2$, with free dihydroxyacetone phosphate in which the labeling has been diluted gives essentially 4- ^{14}C -labeled fructose diphosphate (see Fig. 7 for details of the mechanism). After loss of carbon 1 phosphate, fructose-6-phosphate reacts with TPP and enzyme disulfide to give glycolaldehyde-TPP compound and 4-phosphoerythryl-S-enzyme-SH, the latter four carbon moiety being labeled in carbon 2. Condensation of this phosphoerythryl-S-enzyme-SH with dihydroxyacetone phosphate which by now is labeled in its terminal carbon atom gives 3, 5- ^{14}C labeled sedoheptulose-1, 7-diphosphate.

A reaction of TPP with fructose-6-phosphate would give glycolaldehyde TPP and 4-phosphoerythryl-S-enzyme-SH. Then, condensation of this four carbon moiety with dihydroxyacetone phosphate, by now labeled in the terminal carbon, would give 3,5-¹⁴C-labeled sedoheptulose-1,5-diphosphate.

If this mechanism is correct, we could now understand the long puzzling fact that phosphoglyceraldehyde and erythrose phosphate, alone among the postulated intermediates of the carbon reduction cycle, are seldom seen in tracer experiments, and have been detected in only minute amounts. Of course, these compounds can be produced from the intermediates discussed here. For example, 4-phosphoerythryl-S-enzyme-SH, if allowed to accumulate, would no doubt give disulfide and erythrose-4-phosphate, though this reaction would compete with the hydrolysis to give phosphoerythronic acid and enzyme disulfhydryl. (Note that the proposed pathway of carboxylation does not lead to the formation of any 6 carbon carboxy-sugar moiety. Such a compound has been suggested as an intermediate in the carboxylation reaction²⁹ but the tentative evidence for it³⁰ has not been substantiated.)

Thus far, mechanisms have been discussed for the following steps in the carbon reduction cycle: the carboxylation reaction, the reductive reactions, the isomerization of triose phosphate to give dihydroxyacetone phosphate, the condensations of two triose moieties to give fructose-1,6-diphosphate, and subsequent transketolase like reactions leading to the formation of sedoheptulose-1,6-diphosphate.

Fructose diphosphate, sedoheptulose diphosphate, fructose-6-phosphate, and sedoheptulose-7-monophosphate all are formed very quickly during photosynthesis. Free pools of each of these sugar phosphates exist and these pools are rapidly saturated with radiocarbon during steady state photosynthesis in the presence of $^{14}\text{CO}_2$. It is conceivable that the phosphatase which acts on a phosphate group at the number 1 carbon atoms of these sugars is not located on the multifunctional enzyme unit, but rather is bound on a common structural protein to which the multifunctional enzyme system is also attached. This structural protein may, in fact, be part of the lamellar system of the chloroplasts, and the phosphatase may not be a soluble protein.

At first sight, it might seem tempting to consider the possibility that the ketose diphosphates transfer a phosphoglycolyl moiety directly to a TPP site which could then become the substrate for a carboxylation reaction whose mechanism could be similar to the one just discussed. Fructose and sedoheptulose diphosphates cannot function for the carboxylation reaction in vitro³¹. Even if we suppose that the in vivo enzyme system is more complete and therefore has special properties, it seems likely that the hexose and heptose diphosphates do not function as substrates for the carboxylation reaction. For one thing, the thermodynamics are unfavorable. There is an input of chemical energy to the reactions of the carbon cycle in the phosphorylation of ribulose-5-phosphate to make ribulose-1,5-diphosphate, and again, in the reduction of the carboxylation product to make triose phosphate. However, the driving force for the conversion of triose phosphate to pentose phosphate must come exclusively from the hydrolysis of phosphates on the number one carbon atom, for there are no inputs of chemical energy

by cofactors from the light reaction in this part of the cycle. Calculations show the thermodynamics of the conversion of triose phosphate to pentose phosphate to be quite unfavorable were it not for the energy released by the hydrolysis of sugar diphosphates³². Also, the carboxydismutase reaction on ribulose diphosphate which produces two molecules of phosphoglyceric acid requires that electrons be transferred from the phosphoglyceraldehyde moiety (carbon atoms 3,4 and 5) of ribulose diphosphate to the carboxylated glycolaldehyde moiety (carbon atoms 1 and 2). Thus, the phosphoglycolaldehyde TPP compound is not sufficient, by itself, for the carboxylation reaction. The bound ribulose diphosphate is required. This is also the reason for postulating the opening of the thiazole ring. The opened ring provides the mechanism for the in vitro transfer of electrons to the carboxylated moiety to bring it to the oxidation level of phosphoglyceric acid.

Once sedoheptulose-7-phosphate is formed, it must, therefore, react with the TPP site to give glycolaldehyde TPP and 5-ribonyl-S-enzyme-SH. There must be within the chloroplast an enzyme which reversibly catalyzes the conversion of this last moiety to ribose-5-phosphate and enzyme disulfide, for ribose-5-phosphate is needed for other biosynthetic reactions. For the carbon reduction cycle, the phosphoribonyl-S-enzyme-SH could undergo isomerization analogous to that already discussed for the formation of dihydroxyacetone phosphate from 3-phosphoglyceryl-S-enzyme-SH. However, the resulting ribulose-5-phosphate moiety may well be phosphorylated with ATP and a kinase function to give the

enzyme disulfide and ribulose-1,5-diphosphate. Phosphoriboisomerase and phosphopentokinase have been found to be closely associated with each other and only separable with difficulty from carboxydismutase in fraction I protein from chloroplast protein³³.

For convenience of discussion the enzyme system containing the phosphoriboisomerase and phosphopentokinase will be referred to as 1A. The system containing carboxydismutase (ribulose diphosphate carboxylase) and the proposed carboxyl-phosphorylating-and-reducing system will be termed 1B. The transketolase type function will be called system 2 and the condensing or aldolase system will be referred to as subsystem 3.

To complete the carbon reduction cycle, the glycolaldehyde moieties derived from the transketolase reactions on fructose-6-phosphate and on sedoheptulose-7-phosphate must be condensed by the transketolase subsystem 2 with the 3-phosphoerythryl -S-enzyme-SH to give xylulose-5-phosphate-TPP addition compounds and enzyme disulfide. The xylulose-5-phosphate moiety must be isomerized to ribulose-5-phosphate. Presumably this may occur in subsystem 1A. It may be that xylulose-5-phosphate is transferred from the TPP function of subsystem 2 to the TPP function of subsystem 1B and that in the process the configuration of hydroxyl and hydrogen about the number 3 carbon atom of the sugar moiety is changed, giving rise to ribulose-5-phosphate-TPP addition compound. If so,

this latter compound could then be phosphorylated giving ribulose diphosphate TPP addition compound, after which the reactions already discussed leading to carboxylation and reduction could occur.

In summary, the multifunctional enzyme system just proposed for the carbon reduction cycle of photosynthesis involves three or four subsystems. Two kinds of functions for the attachment of substrate moieties occur. One is a TPP function which attaches substrate moieties at its number 2 position. The other is an enzyme disulfide (reduced form disulfhydryl) function which attaches substrate moieties to one of its sulfurs and is capable of undergoing oxidation reduction.

These functional points of attachment for substrate must be integrated with the specific enzymic machinery represented by the postulated subsystems with their specific enzymic activities. The points of substrate attachment serve as handles holding the substrate while it is acted upon by the specific enzyme activities. Also, they permit the substrate moiety to be transferred from one enzymic position to another. The mechanical details of such types of attachment and transfer have been discussed by Lynen¹² and others.

Probably there are at least two types of TPP functions. One type would be involved in the transketolase reactions and reacts with the sugar monophosphates. The other type, involved in the carboxylation and reductive reactions, is capable of reacting with ribulose diphosphate. There may be three types

of enzyme disulfhydryl (oxidized form-disulfide) functions.

One would be specific for the acceptance of phosphoglyceryl moieties formed from the carboxylation reaction. A second would be specific for accepting 4-phosphoerythryl moieties from transketolase reaction on fructose-6-phosphate, and a third would be specific for accepting the 5-phosphoribonyl moiety from the transketolase reaction on sedoheptulose-7-phosphate.

Only the first of these three should be capable of accepting electrons from the light reaction.

Before turning to direct chemical or biochemical evidence for the mechanisms just postulated, let us see how such a mechanism could account for some of the known kinetic data on the labeling of intermediates of the carbon reduction cycle during photosynthesis in the presence of $^{14}\text{CO}_2$. The irreversibility of the reduction of carbon to sugar phosphates at the site of the carbon reduction cycle has already been mentioned. In terms of the mechanism just proposed, this irreversibility could be accounted for by postulating that the oxidation of the phosphoglyceryl-S-enzyme-SH is not enzyme mediated, and therefore occurs only slowly. This compound was formed in the postulated mechanism by the transfer of phosphoglyceryl moieties from the carboxylation and reduction reactions to enzyme disulfhydryl. The enzyme disulfhydryl in turn was formed by the reduction of enzyme disulfide by electrons from the light reaction of photosynthesis. Enzymic oxidation of the enzyme disulfide or of a precursor electron donor from the

light reaction must of necessity be blocked. Otherwise, electrons from the primary light reaction would be short circuited back to the oxygen producing moiety from the water splitting reaction of photosynthesis. Thus, the carbon reduction process is accomplished by an essentially non-reversible process.

One can expect the small and temporary oxidation of some newly incorporated radioactivity in sugar phosphates that was discussed earlier¹⁴. When the light is turned off, there must exist a certain concentration of enzyme disulfide function (oxidized form). This can react with fructose-1,6-diphosphate by a reversal of the reactions shown in Fig. 7 to give dihydroxyacetone phosphate and phosphoglyceryl-S-enzyme-SH, which hydrolyzes non-enzymically to phosphoglyceric acid and enzyme disulfhydryl. Once the enzyme disulfide function has all been converted to enzyme disulfhydryl by this mechanism further oxidation can occur only by the slow thermal oxidation of the disulfide.

One piece of experimental chemical evidence for the proposed mechanism is the formation of glycolic and of phosphoglycolic acids during short periods of photosynthesis at low CO₂ pressures as a function of oxygen pressure³⁴. After two minutes photosynthesis by Chlorella in the presence of ¹⁴C-labeled bicarbonate in N₂ and in O₂, a 25-fold stimulation of the production of radioactive glycolic acid and a two-fold stimulation of the production of radioactive phosphoglycolic acid were observed in the experiment with O₂ as compared with the experiment with N₂. This result strongly suggests the oxidation of the two carbon moiety derived from carbon atoms 1 and 2 of the sugar phosphates. Calvin and Bassham³⁵ suggested that the formation of glycolic acid during photosynthesis occurs via the oxidation of the glycolaldehyde

TPP compound formed by the transketolase enzyme system, following the transfer of the glycolaldehyde moiety to a disulfide such as lipoic acid. The glycolyl moiety might just as well be transferred to the enzyme disulfide function proposed here. The action of the oxygen may well be the non-enzymic oxidation of this disulphydryl function to disulfide.

The formation of phosphoglycolic acid may be taken as evidence for the existence of a phosphoglycolaldehyde moiety such as the phosphoglycolaldehyde TPP addition compound postulated for the carboxylation reaction. The acceleration of glycolic acid formation by low CO₂ pressures could result from the build-up of phosphoglycolaldehyde TPP compound due to a decreased rate of the carboxylation reaction. It is noteworthy that glycolic acid formation, even though it is an oxidative reaction, does not appear to occur in the dark. This is quite understandable in terms of the discussion above, where it was suggested that in the dark the enzyme disulfide function becomes converted to the enzyme disulphydryl as a mechanism which blocks the further oxidation of sugar phosphates to phosphoglyceric acid. In the light, some enzyme disulfide becomes available due to condensation of phosphoglyceryl-S-enzyme-SH with dihydroxyacetone phosphate.

Another reaction of the phosphoglycolaldehyde moiety which perhaps does occur in the dark, is its conversion via a phospho-ketolase type reaction to acetyl phosphate. The indirect evidence for this supposition is the acceleration of the conversion of intermediates of the carbon reduction cycle to compounds of the Krebs cycle and particularly to glutamic acid when the light is turned off. It was at one time suggested¹ that there was a light

block on the oxidation of pyruvic acid to acetyl CoA. This light block was thought to operate by the primary reductant from the light reactions of photosynthesis keeping lipoic acid in the disulphydryl form when the light was on. In the dark, the lipoic acid was thought to go to the disulfide form.

However, the kinetics of carbon fixation which recently were followed when radiocarbon was only added just at the moment of turning off the light¹⁴ showed that under these conditions there was no significant conversion of phosphoglyceric acid to glutamic acid. In fact, there was no significant formation of labeled glutamic acid. This result therefore suggests that the radiocarbon which finds its way into glutamic acid when the light is turned off comes from acetyl phosphate derived from the sugar phosphates of the carbon reduction cycle. These sugar phosphates are not significantly labeled in the experiment in which the radiocarbon is added only at the moment the light is turned off.

We may view the accelerated release of acetyl phosphate upon turning off the light in the following way: When the light is turned off, the phosphorylation of bound ribulose-5-phosphate stops, since no further generation of high energy phosphate is occurring. For this reason, it might be expected that this bound ribulose-5-phosphate and in turn glycolaldehyde TPP compound would accumulate. However, its conversion to glycolic acid is also blocked for the reason just discussed, namely that the enzyme disulphydryl can no longer be oxidized to the required disulfide form. Consequently, the principal metabolic pathway open to the

glycolaldehyde TPP addition compound is its conversion to acetyl phosphate via phosphoketolase. It is well known³⁶ that acetate and presumably acetyl phosphate or acetyl CoA is very rapidly incorporated in algae into glutamic acid and intermediates of the Krebs cycle as well as into fats in fatty acids.

It is worth noting that when the light is turned off during a steady state light-dark transient study of previously carbon labeled algae, there is no increase in the level of ribulose-5-phosphate^{14,37}. The levels of all of ^{the} / pentose phosphates are quite small and there is some doubt that any significant amount of ribulose-5-phosphate has been observed. Small amounts of ribulose-5-phosphate plus ribose-5-phosphate were reported, but more recent studies (unpublished) indicate that the pentose monophosphate other than ribose-5-phosphate is probably xylulose-5-phosphate. This observation suggests that just as was proposed in the mechanism above, ribulose-5-phosphate may not exist as a free compound but rather may be represented only by an enzyme bound ribulose-5-phosphate moiety which is phosphorylated on the enzyme.

In summary, it may be said that the proposed multifunctional enzyme system explains a large number of experimental data obtained from studies of the kinetics of formation of radioactive compounds during photosynthesis. More direct proof of the proposals made here must await biochemical studies of the enzymes isolated from plant materials.

REFERENCES

1. M. Calvin and P. Massini, *Experientia* VIII, 445 (1952).
2. J.A. Bassham, A.A. Benson, L.D. Kay, A.Z. Harris, A.T. Wilson and M. Calvin, *J. Am. Chem. Soc.* 76, 1760 (1954).
3. J.A. Bassham and M. Calvin, *The Path of Carbon in Photosynthesis*, Prentice-Hall, Englewood Cliffs, N.J., 1957.
4. J.A. Bassham and M. Kirk, *Biochim. et Biophys. Acta* 43, 447 (1960).
5. A.T. Wilson and M. Calvin, *J. Am. Chem. Soc.* 77, 5948 (1955).
6. W. Vishniac and S. Ochoa, *Nature* 167, 768 (1951).
7. A. Peterofsky and E. Racker, *Plant Physiol.* 36, 409 (1961).
8. G. Richter, *Naturwissenschaften* 41, 604 (1959).
9. C.A. Fewson, M. Al-Hafidh and M. Gibbs, *Plant Physiol.* 37, 402 (1962).
10. C.A. Fewson, P.K. Kindel and M. Gibbs, *Plant Physiol.* 36, suppl. IX (1961).
11. Mary Stiller, *Ann. Rev. Plant Physiol.* 13, 151 (1962).
12. F. Lynen, *Fed. Proc.* 20, (4), 934 (1961).
13. A.A. Benson, S. Kawaguchi, P.M. Hayes and M. Calvin, *J. Am. Chem. Soc.* 74, 4477 (1952).
14. J.A. Bassham and Martha Kirk, *Plant and Cell Physiol.*, in press.
15. O. Kandler and M. Gibbs, *Plant Physiol.* 31, 411 (1956).
16. A. San Pietro and H.M. Lang, *Science* 124, 118 (1956).
17. A. San Pietro and H.M. Lang, *J. Biol. Chem.* 231, 211 (1958).
18. K. Tagawa and D.I. Arnon, *Nature*, in press.
19. A.G. Datta and E. Racker, *Arch. Biochem. Biophys.* 82, 489 (1959).
20. A.G. Datta and E. Racker, *J. Biol. Chem.* 236, 624 (1961).
21. R. Breslow, *J. Am. Chem. Soc.* 80, 3179 (1958).
22. R. Breslow, *J. Cellular Comp. Physiol.* 54, Suppl. 1, 100 (1959).

23. L.O. Krampitz, J. Cellular Comp. Physiol. 54, Suppl. 1, 101 (1959).
24. H. Holzer, R. Katterman and D. Busch, Biochem. Biophys. Res. Comm. 7, 167 (1962).
25. I.C. Gunsalus, L.S. Barton and W. Gruber, J. Am. Chem. Soc. 78, 1763 (1956).
26. H. Holzer and K. Beucamp, Biochim. Biophys. Acta 46, 225 (1961).
27. L.J. Reed, Adv. in Enzymol. 18, 319 (1956).
28. T. Yusa, Plant and Cell Physiol. 2, 471 (1961).
29. M. Calvin, Fed. Proc. 13, 697 (1954).
30. V. Moses and M. Calvin, Proc. Nat. Acad. Sci. 44, 260 (1958).
31. A. Weissbach, B.L. Horecker and J. Hurwitz, J. Biol. Chem. 218, 795 (1956).
32. J.A. Bassham, J. Theor. Biol., in press.
33. G. van Noort, Doctoral Dissertation, 1962, University of California, Los Angeles.
34. J.A. Bassham and M. Kirk, Biochim. Biophys. Res. Comm., in press.
35. J.A. Bassham and M. Calvin, The Photosynthesis of Carbon Compounds, W.A. Benjamin, Inc., N.Y., 1962.
36. D.C. Smith, J.A. Bassham and M. Kirk, Biochim. Biophys. Acta 48, 299 (1961).
37. J.A. Bassham, K. Shibata, K. Steenberg, J. Bourdon and M. Calvin, J. Am. Chem. Soc. 78, 4120 (1956).

This report was prepared as an account of Government sponsored work. Neither the United States, nor the Commission, nor any person acting on behalf of the Commission:

- A. Makes any warranty or representation, expressed or implied, with respect to the accuracy, completeness, or usefulness of the information contained in this report, or that the use of any information, apparatus, method, or process disclosed in this report may not infringe privately owned rights; or
- B. Assumes any liabilities with respect to the use of, or for damages resulting from the use of any information, apparatus, method, or process disclosed in this report.

As used in the above, "person acting on behalf of the Commission" includes any employee or contractor of the Commission, or employee of such contractor, to the extent that such employee or contractor of the Commission, or employee of such contractor prepares, disseminates, or provides access to, any information pursuant to his employment or contract with the Commission, or his employment with such contractor.

