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# Ernest O. Lawrence Radiation Laboratory

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J. A. Bassham

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# KINETIC STUDIES OF THE PHOTOSYNTHETIC CARBON REDUCTION CYCLE[1]

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## Introduction

The problem of the carbon reduction pathway of photosynthesis is considered to be solved by some plant physiologists (1). The pathway which is often accepted as being correct is the reductive pentose phosphate cycle, Calvin cycle, or, as I shall refer to it, the photosynthetic carbon reduction cycle (PSCR) (2). This is the only such pathway proposed in recent years which is essentially complete in its specification of intermediates and reaction sequences.

In the PSCR cycle, the first stable carboxylation product is 3-phosphoglyceric acid (PGA) (3). All the remaining stable intermediates are sugar phosphates and diphosphates, with carbon skeletons from three to seven carbon atoms in length. In the simplest terms, the PSCR cycle consists of four stages: 1) ribulose-1,5-diphosphate (RuDP) is carboxylated to give two molecules of PGA; 2) PGA is reduced to triose phosphate; 3) a series of reactions convert five triose phosphate molecules to

give three pentose phosphate molecules; 4) the pentose phosphate molecules are then phosphorylated to give RuDP. Carbon which enters this cycle as carbon dioxide is later "drained off" as reduced carbon in the form of PGA or sugar phosphates by secondary photosynthetic pathways. These pathways lead ultimately to the synthesis of fats, proteins, carbohydrates and other products.

There are some dissenters from the acceptance of the PSCR cycle. Stiller (4) has written an interesting and provocative review of the path of carbon in photosynthesis. She argues that the PSCR cycle is incorrect, and proposes an alternative path (in which the first steps are not completely defined).

Most of the differences of opinion regarding the cycle revolve around the interpretation of the available experimental data, rather than around the correctness of the data itself. We have reviewed the evidence used in formulating the cycle (5,6). In the present article, I shall discuss some of the questions which have been posed regarding the interpretation of the data, and then shall turn to a consideration of recent experimental developments which suggest some possible mechanisms of reactions

of the carbon reduction pathway. The probability of the operation in vivo of some organized enzymic system will be considered. Except for the formation of glycolic acid, secondary pathways leading from the basic carbon reduction cycle will not be discussed. These paths, leading to malic acid, amino acids, sucrose and other carbohydrates, and various other products of photosynthetic carbon reduction have been reviewed elsewhere (6).

I shall neglect the many excellent studies with whole and broken chloroplast preparations. Such studies are most valuable for revealing many of the biochemical capabilities of the photosynthetic apparatus. Owing to the greatly diminished efficiency of these systems for $CO_2$ reduction, it is my opinion that little can be learned from them concerning the kinetics of carbon reduction in vivo.

Two lines of evidence have been used in connection with the elucidation of the carbon reduction pathway. The primary line of evidence consists of the kinetics of the labeling of

compounds in the living cell following the introduction of labeled substrate. No other tool has been found for obtaining information about the identity of the intermediates in the primary carbon reduction pathway or the sequence of reactions which link them. One can and must criticize the method and be conscious of its limitations. If we were to disregard this evidence, however, we would know almost nothing about the pathway.

The second and supporting line of evidence comes from the study of enzymic activity of soluble and particulate fractions isolated from broken photosynthetic cells. The demonstration of such biochemical capabilities in isolated enzymes gives us added confidence in a reaction which we have been led to postulate as occurring in a living system on the basis of kinetic data. The failure of attempts to isolate any or sufficient enzymic activity for a given proposed reaction is not an overwhelming argument against the occurrence of that reaction as a step in an <u>in vivo</u> metabolic pathway.

The fact is that considerable success has attended the efforts to isolate from photosynthetic tissue of a variety of plants the various enzymic activities required by the PSCR cycle. This evidence has been reviewed by Vishniac et al. (7). Stiller (4) has reviewed most of the more recent enzymic studies. Where there has been reported an inadequate activity for a given reaction from a given organism, we should see what this might tell us about the accomplishment of the reaction in vivo. Such discrepancies suggest that the organism may have something in its makeup which causes the disruption or loss of enzymic activity upon attempts to isolate such activity. The proper interpretation of such effects can conceivably be useful in understanding the mechanism of the enzymic system in vivo.

## The Carbon Reduction Cycle In Vivo

The carbon reduction cycle or the PSCR cycle is shown in Figure 1. The reactions are the same as proposed previously (2,5,6) but the cycle has been redrawn to emphasize the relations between ketose phosphates, the glycolaldehyde thiamine pyrophosphate addition compound and glycolic acid (6). The approximate labeling

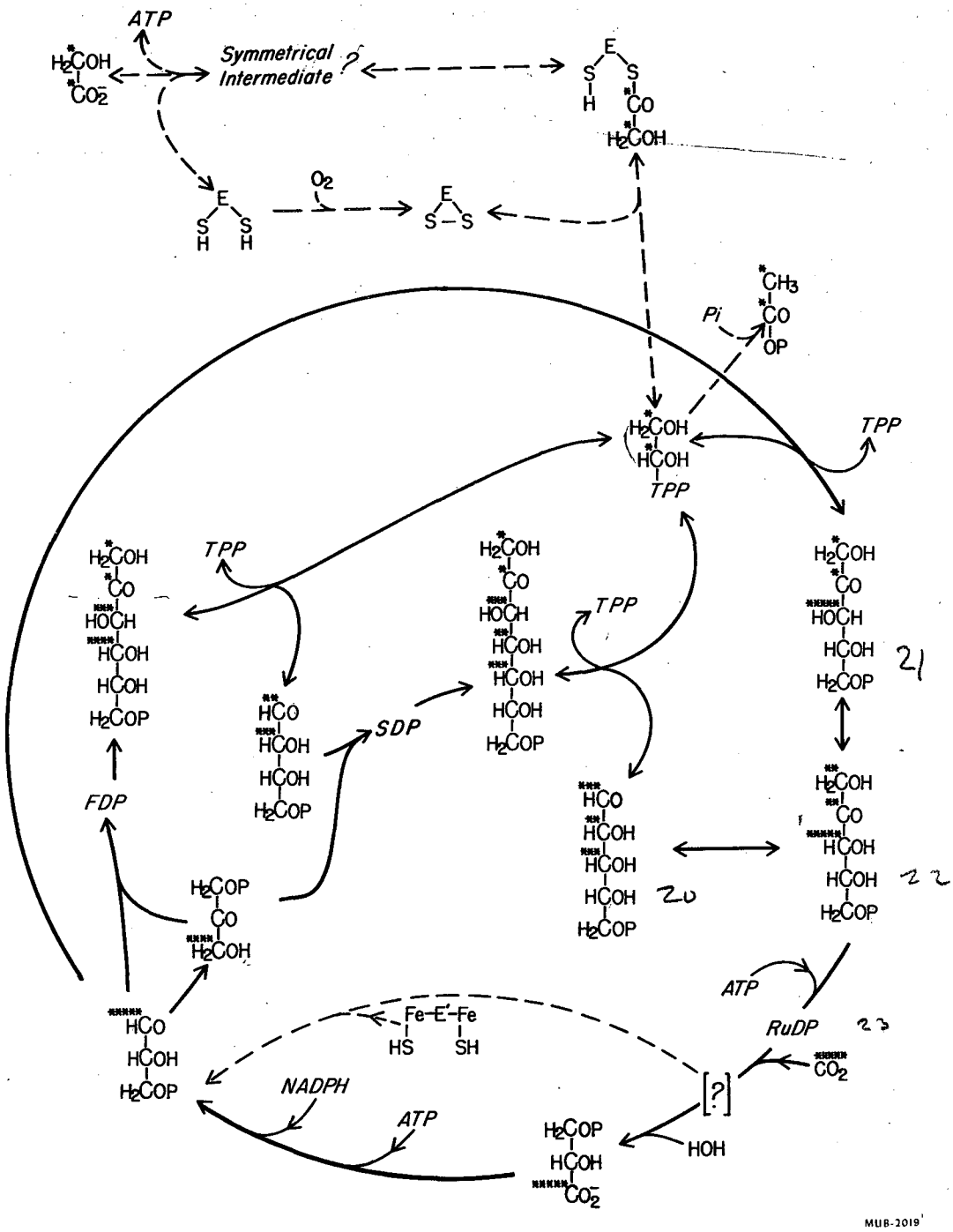


Fig. 1. The photosynthetic carbon reduction cycle (PSCR cycle). Abbreviations include: FDP, fructose-1, 6-diphosphate; SDP, sedoheptulose-1, 7-diphosphate; RuDP, ribulose-1, 5-diphosphate; TPP, thiamine pyrophosphate; E, E', unspecified enzymes or proteins. The $E'(FeSH)_2$ symbolizes a reduced ferredoxin-type electron carrier with a potential of -0.4 v. The asterisks denote the order of labeling (not the accurate magnitude) of various carbon atoms of sugar phosphates, and other intermediates, following a short period (such as 10 sec) of photosynthesis with $^{14}CO_2$. Depending on pool sizes, particularly that of triose phosphates, asymmetry of labeling in hexose and heptose phosphates may be more or less than shown.

pattern after a few sec photosynthesis with $^{14}CO_2$ is indicated by asterisks. Also shown is the hypothetical *in vivo* reduction of the carboxylation product by high negative potential (-0.4 v) reducing agents, such as reduced ferredoxin. I shall review the basic kinetic arguments relating to this scheme. At the same time some new discussion of the kinetics of published results will be presented. It will be assumed that the techniques of photosynthetic studies with $^{14}CO_2$ as a tracer (5) are well known to the reader.

3-Phosphoglyceric acid. 3-Phosphoglyceric acid (PGA) was found to be the most prominently labeled compound after very short periods of photosynthesis in the presence of $^{14}CO_2$ (3,8). Kinetic studies showed that when the percentage of total radiocarbon fixed was plotted as a function of time of photosynthesis with $^{14}CO_2$, the percentage found in PGA when extrapolated to zero time was more than 70% (2) and in some experiments nearly 100% (3).

In those experiments where the extrapolated percentage in PGA at zero time was about 70%, much (10-15%) of the remainder was found distributed among several sugar phosphates, particularly

fructose and sedoheptulose phosphates. From the standpoint of known chemical or biochemical reactions, these substances are not likely first products of a carboxylation reaction. It was supposed that they were derived from the primary carboxylation product, which was thought to be PGA.

In these same experiments, other small amounts of $^{14}C$ were found during the shortest times in malic acid, which was presumed therefore to be a product of a carboxylation reaction. For a time it was thought that malic acid might be an intermediate in a regenerative carbon reduction cycle. However, studies with malonic acid as an inhibitor (9) showed that even though the formation of malic acid could be prevented, the operation of the carbon reduction cycle, as evidenced by the incorporation of $^{14}C$ into the α and β carbons of PGA was not impeded.

Such information was obtained from the chemical degradation of phosphoglyceric acid (9). A kinetic study of the appearance of radiocarbon in the individual atoms of phosphoglyceric acid showed that radiocarbon appeared first in the carboxyl carbon and later equally in the α and β carbon atoms. After 60 sec of photosynthesis

in the presence of $H^{14}CO_3^-$, the distribution of $^{14}C$ among carboxyl, α, and β carbon atoms of PGA was about in the ratio 2:1:1, respectively.

This finding had an important consequence. It suggested that the α and β carbon atoms of PGA are not formed de novo from $CO_2$, but rather that they are derived from the carboxyl carbon atom via a cyclic process. Only the carboxyl carbon atom appeared to come directly from $CO_2$ via a carboxylation reaction.

The possibility that unstable intermediates proceed the observed stable products of carbon fixation long has been recognized. A careful search (10,11) failed to reveal the existence of such early unstable products to date. While by definition the possible existence of such unstable intermediates never can be eliminated completely, certain limitations can be set upon the concentration and stability of such substances by the experimental results thus far obtained.

We have been able to show by means of kinetic studies (12)

of the photosynthetic fixation of $^{14}CO_2$ during steady state photosynthesis in Chlorella pyrenoidosa that there could be no more than 1.5 micromoles of carbon per $cm^3$ of Chlorella cells, lying between the introduced radioactive carbon dioxide and stable products of fixation. Under the experimental conditions used this was equivalent to about 5 sec of photosynthesis. In our opinion the most reasonable assumption as to the nature of most of this 1.5 micromoles of $CO_2$ is that it represents a pool of dissolved carbon dioxide and bicarbonate inside the Chlorella cells.

In the same report, we listed "steady state" concentrations of a number of intermediate compounds of the carbon reduction cycle. These concentrations of actively turning over pools were obtained by the technique of allowing them to become "saturated" with $^{14}C$ during steady state photosynthesis with $^{14}CO_2$ of constant specific radioactivity (S) (12). From the measured $^{14}C$ in each compound at saturation (A), (C) the amount of carbon in each reservoir (in units such as micromoles/$cm^3$ wet packed algae) can be calculated according to the formula $C = A/S$. A knowledge of such pool sizes and of the net rate of flow of material can be used in

predicting the rates of labeling of intermediate compounds and of specific carbon atoms within the intermediate compounds under various assumptions as to the biochemical pathways involved.

For example Stiller (4) proposed that carbon reduction in photosynthesis involves a de novo synthesis of a diose moiety from $CO_2$. This diose is then supposed to condense with triose phosphate to give RuDP which would then undergo carboxylation to give two molecules of PGA. The PGA would then be reduced to triose phosphate, completing a small cycle.

In the experiment just quoted (12) the rate of flow ($R_2$) of carbon through the diose would be 2/3 of 12, or 8 micromoles/min· $cm^3$ algae. The pool size of RuDP in the same experiment was only 0.36 micromoles, or 0.07 micromoles per carbon atom position, while the corresponding values for PGA were 3.0 and 1.0, respectively, and for triose phosphate they were 0.8 and 0.27.

It is a consequence of Stiller's proposal that $^{14}C$ introduced by carboxylation of RuDP would pass through PGA carboxyl, triose number one carbon, RuDP carbon atom number 3, and then PGA

carboxyl again. The $^{14}C$ introduced through "diose" would pass through the "unstable pool", thence through RuDP carbon atoms 1 and 2, the α and β carbon atoms of PGA, carbon atoms 2 and 3 of triose phosphate, carbon atoms 4 and 5 of RuDP and back into the PGA α and β carbon atoms.

Thus the carboxylation carbon passes through one set of carbon atoms whose total concentration we can denote by $C_1$ and is equal to 1.0 + 0.27 + 0.07 = 1.34 micromoles. The "diose" carbon passes through a different set of carbon atom positions which we can denote by $C_{2,3}$. Including the unstable pool (whose upper limit in size is 1.5 micromoles), $C_{2,3}$ is equal to 1.5 + 0.14 + 2.0 + 0.54 + 0.14 = 4.32 micromoles.

A precise calculation of the degree of labeling, particularly at times less than 30 sec, would require consideration of each component pool in $C_1$ and $C_{2,3}$ and would be rather complicated, due to the cyclic nature of the process. We can obtain a reasonably good approximation of the average degree of saturation by 60 sec of $C_1$ and of $C_{2,3}$ by treating them as single pools. The degree of saturation of $C_1$ is then given by $x_1 = 1 - e^{-R_1 t/C_1}$ and at t = 1 min it is 0.95.

By a similar calculation the degree of saturation of $C_2$ is 0.84. It then follows that the degree of saturation of the entire PGA pool at 1 min would be $[0.95 + 2(0.84)]/3 = 0.88$. It can be calculated from experimental data in reference 12 (at 59 sec) as $2.29/3 = 0.78$. The degree of saturation of the ribulose diphosphate, by calculation from these $C_1$ and $C_{2,3}$ values would be $[4(0.84) + 0.95]/5 = 0.86$, whereas the calculation from the experiment is (at 59 sec) $0.225/.36 = 0.625$.

A slower rate of saturation of PGA and of RuDP would result from the operation of the PSCR cycle because of the larger total pool of carbon in the PSCR cycle. The total concentration of carbon in intermediates of the PSCR cycle in the above experiment is $C = 12.2$. When $R = 12$ and $t = 1$, $x = 1 - e^{-1} = 0.632$. This average value for all the carbon positions in the cycle is thus in good agreement with the experimentally calculated value for RuDP (0.625). The carboxyl carbons of PGA will be nearly saturated by one min so the estimated value for the three carbon atoms of PGA would be $[1 + 2(0.632)]/3 = 0.755$, also close to the experimental value of 0.78. These agreements between calculated

and experimental results probably are better than should be expected, considering the approximations used. They do illustrate that some cyclic process, involving intermediates or substances in rapid equilibrium with intermediates, whose total concentrations are about those of substances involved in the PSCR cycle, is required to fit the available kinetic data.

In her review (4), Stiller asserted (in support of her proposal) that our studies (12) indicate that "the carbon atoms of pentose phosphate attain a higher specific activity than those of the phosphoglyceric acid from which they are presumably derived". We found and reported precisely the opposite. From the beginning of the period of steady state synthesis with $^{14}CO_2$ until saturation of the intermediates the specific activity of the PGA was always considerably higher than that of RuDP.

If the carboxylation of RuDP results in the formation of two molecules of PGA, then the carboxyl group of 1 of every two PGA molecules will contain the newly incorporated $^{14}C$.

To test this model, we subtracted from the total PGA radiocarbon an amount of $^{14}C$ radioactivity which would correspond to the radio-

activity expected in this carboxyl if the model were correct. Since this carboxyl group would rapidly saturate if the model were correct, this involved subtracting 1/2 x 1/3 = 1/6 of the saturation level of radiocarbon in PGA after about 30 sec. The remaining $^{14}C$ which would have to be derived from the RuDP carbon atoms was compared with the $^{14}C$ in RuDP. The remaining, or residual, PGA carbon atoms were found to be labeled to a higher degree of saturation than the average of the five atoms of RuDP, suggesting that this model (2 molecules of PGA per carboxylation) was incorrect.

Using a different model in which only one PGA molecule was formed, and in which it was formed from the newly incorporated $^{14}CO_2$ and carbon atoms 1 and 2 of RuDP, a similar calculation showed that the residual carbon atoms of PGA were not labeled more rapidly than the average of the five RuDP carbon atoms until after 50 sec. In this case there was no contradiction between model and data even after 50 sec, since we know that carbon atoms 1 and 2 of RuDP are more quickly labeled than the carbon atoms 3,4 and 5 (2).

From this data and reasoning, we concluded that the carboxylation of RuDP leads to only one molecule of PGA in equilibrium with the PGA pool. The other three carbon atoms from RuDP appear to have been

converted either to a form of bound PGA not in equilibrium with the pool, or to some other molecule. We speculated that if the in vivo reaction were reductive, the other molecule might be triose phosphate.

It was noted earlier that the radioactivity in PGA does not always extrapolate to 100% at zero time (2). Sometimes such extrapolation gives 10-15% radiocarbon in sugar phosphates at zero time. This finding suggests that it is not only the three carbon moiety derived from carbons 3,4 and 5 of RuDP which may not be in equilibrium with the PGA pool. It appears that some of the PGA labeled with the newly incorporated $^{14}C$ in the carboxyl group is also bound, perhaps to an enzyme, and converted to sugar phosphates without freely equilibrating with the PGA pool.

Reduction of PGA to sugar phosphates. Calvin and Benson (13) proposed that PGA is reduced to triose phosphate and that triose phosphate then condenses to make hexose phosphates. This proposal was supported by their identification of PGA, triose phosphate, and hexose phosphates as labeled products following short periods of photosynthesis in the presence of $^{14}CO_2$ (14). The reduction of PGA to hexoses via a pathway resembling the reverse of glycolysis was

indicated by degradation of hexose and of PGA from the same experiment (3). For example, after 15 sec photosynthesis by barley the distribution of radiocarbon in the carboxyl and α and β carbons of PGA was roughly 50%, 25% and 25% respectively, while that in sucrose $C_3$ and $C_4$, $C_2$ and $C_5$, and $C_1$ and $C_6$ was also 50%, 25% and 25%, respectively.

This degradation of hexose derived from sucrose did not differentiate between the carbon atoms 1,2 and 3 of the hexose as compared with carbon atoms 4,5 and 6. The Gibbs effect, had it been present, would not have been observed. Kandler and Gibbs (16) later found that following photosynthesis with $^{14}CO_2$, both glucose phosphate and the glucose moiety of starch were asymmetrically labeled. Carbon atom 4 was more highly labeled than carbon 3, while carbon atoms 1 and 2 were more highly labeled than carbons 5 and 6.

It is not difficult to propose a reasonable explanation of this Gibbs effect which is consistent with the PSCR cycle (2,5). The difference between 3 and 4 could be predicted in terms of the PSCR cycle on the basis of the measurable pool of dihydroxyacetone phosphate. This pool dilutes the tracer coming into it when phosphoglyceraldehyde is converted to dihydroxyacetone

phosphate. Dihydroxyacetone phosphate then reacts with newly formed phosphoglyceraldehyde which has a higher specific activity. This reaction results in the formation of hexose more highly labeled in carbon atom 4, derived from the phosphoglyceraldehyde, than in carbon atoms 3, derived from the dihydroxyacetone phosphate (2) (See Fig. 1).

The higher labeling of carbon atoms 1 and 2 as compared with 5 and 6 can be readily explained in terms of the reversibility of the transketolase reaction (5) (See Fig. 1). Careful consideration of both of these explanations will show that the size of the effect or indeed its existence will depend to a large extent on pool sizes and the rate of the net forward reaction as compared with the rate of the reverse reactions. It is not surprising therefore if sometimes the Gibbs effect is observed and sometimes it is not.

It is true that we have reported (2) that after 5.4 sec photosynthesis with Scenedesmus, the fructose phosphate was symmetrically labeled with most of the radioactivity being in carbon atoms 3 and 4. It will be noted, however, that sedoheptulose phosphate obtained from that same sample contained most of its radioactivity equally

distributed between carbons 3,4 and 5. Since carbon atoms 4 and 5 of sedoheptulose phosphate are derived from carbons 3 and 4 of fructose phosphate (2), this result is self-consistent.

In the same report we found that in sedoheptulose phosphate from soy bean leaves which had been exposed to $^{14}CO_2$ for very short times (est. 0.4 sec), carbon atom 4 contained only 8% of the radiocarbon while the total of carbons 1,2 and 3 was 33% and the total of carbons 4,5 and 6 was 57%. It is evident (from other results in that report) that carbon 3 by itself contained most of the 33% and carbon 5 contained 57% minus 8%, or 49%. Thus, in that experiment, the asymmetry in carbon atoms 4 and 5 derived from carbon atoms 3 and 4 of fructose phosphate according to the PSCR cycle was observed. However, in that particular sample, only the two halves of the hexose were compared in degradation studies and were found to be approximately equal. If carbon atom 1 and 2 are more labeled than carbon 5 and 6, while carbon atom 3 is less labeled than carbon atom 4, the two halves (1 + 2 + 3 and 4 + 5 + 6) of hexose can be equally labeled fortuitously.

While Stiller did not discuss the labeling of sedoheptulose

phosphate which would result if her hypothesis were correct, it can be readily predicted. It is a consequence of her hypothesis that carbon atoms 1 and 2 of pentose phosphate must always be equally labeled. Sedoheptulose phosphate formed by transketolase from such a pentose would therefore always have carbon atoms 3 and 4 equally labeled. This requirement is clearly contradicted by our published degradation of sedoheptulose from the 0.4 sec soy bean leaf (2) (See above).

In the study of the kinetics of $^{14}C$ labeling of intermediates during steady state photosynthesis (12,15,17,18), two classes of compounds can be distinguished. One class of compounds includes those which become saturated with $^{14}C$ within 3 or 4 min after its introduction as $^{14}CO_2$. This saturation is the result of the fact that their pool sizes are relatively small and that they are for the most part intermediates in the basic carbon reduction cycle so that the net rate of flow of carbon through these compounds is large. All of the intermediates postulated for the PSCR ¹ cycle plus phosphoenolpyruvic acid and glucose-6-phosphate fall in this category.

A second class of compounds includes such substances as malic acid, sucrose, alanine, etc., which do not become saturated with $^{14}C$ for many min after its introduction as $^{14}CO_2$. It is clear that these compounds cannot lie in the cyclic path of carbon reduction. No intermediate in the cyclic path of carbon reduction can become saturated before any other intermediate. It is true that a substance such as phosphoenolpyruvic acid or glucose-6-phosphate can be in such rapid equilibrium with an intermediate in the cycle that it can saturate at essentially the same rate as intermediates in the cycle.

It would be possible that by this criterion fructose-1,6-diphosphate is not an intermediate in the cycle (4) but merely a substance in rapid equilibrium with such intermediates as triose phosphate. However, if this were the case, one must dismiss the argument that there is an inadequate amount of enzymic activity such as aldolase to form fructose-1,6-diphosphate at a rate consistent with its being an intermediate in the cycle (4). As much enzymic activity is needed for the rapid equilibration of a compound with intermediates in the cycle as would be required for the substance to be itself an intermediate in the cycle.

As we pointed out ten years ago (2) there is little difference in the rates of labeling among the various intermediates in the cycle except PGA. The more rapid labeling of PGA is of course due to its carboxyl group which contains the most recently incorporated carbon. Thus except in the case of PGA, the positions of intermediates in the cycle were not determined by sequence of labeling of the intermediates as has been suggested (4). The sequence of cycle intermediates was determined by degradation studies, transient changes in concentrations of labeled compounds accompanying sudden light-dark or high $CO_2$-low $CO_2$ conditions, and a consideration of known biochemical reactions (2,5).

Conversion of triose phosphate to pentose phosphate. The degradation data supporting the steps given by the PSCR cycle for the conversion of five triose phosphate molecules to 3 pentose phosphate molecules (see Fig. 1) have been amply discussed before (2,5,6). A detailed diagram of the distribution of label through the PSCR cycle was recently published (18).

It has been suggested sometimes that sedoheptulose-1,7-diphosphate is not an intermediate in the cycle and that sedoheptulose-7-phosphate

is formed by a transaldolase reaction (7). Sedoheptulose-1,7-diphosphate, like fructose-1,6-diphosphate and RuDP is rapidly labeled and quickly saturated in a manner similar to other postulated intermediates of the PSCR cycle, during steady state experiments with $^{14}CO_2$. Moreover, like these other diphosphates, sedoheptulose diphosphate shows a rapid transient drop in concentration when the light is turned off (20). Recently (21) labeled sedoheptulose diphosphate has been observed to undergo a rapid transient increase upon the introduction of the inhibitor 6-methyl lipoic acid. At the same time, there is a rapid drop in labeled sedoheptulose-7-phosphate. This behavior indicates that the monophosphate is formed from sedoheptulose-1,7-diphosphate and that both are carbon cycle intermediate compounds. Moreover, transaldolase activity is more difficult to find in adequate amounts in some green tissues than aldolase activity (22).

Phosphorylation of ribulose-5-phosphate. Kinetic studies of transients in labeling pattern following steady state photosynthesis in the presence of $^{14}CO_2$ point to a light-stimulated phosphorylation of ribulose-5-phosphate to make RuDP. As mentioned

earlier, Calvin and Massini (15) found that upon turning off the light, the concentration of ribulose diphosphate dropped rapidly to zero in contrast to the behavior of most of the other sugar phosphates. The dark-induced drop to zero in RuDP concentration has been observed in subsequent studies (20,23), but in one of these (20) fructose diphosphate concentrations also dropped close to zero. The drop in concentration of RuDP was attributed to a cessation in its formation in the dark from ribulose-5-phosphate by a kinase reaction requiring ATP produced by light reactions. At the same time, the carboxylation reaction requiring no light-produced cofactors was presumed to continue in the dark, thus depleting the RuDP.

The carboxylation reaction. Calvin and Wilson (17) found that when the $CO_2$ pressure was lowered to a few thousandths of a per cent following a period of photosynthesis with $^{14}CO_2$, the most noticeable transient changes were a rapid rise in $^{14}C$-RuDP and a rapid drop in $^{14}C$-PGA. This strongly suggests that RuDP is the carboxylation substrate in the reaction leading to the formation of PGA. It was mentioned earlier that several studies (3,20,23) of the

transient behavior of labeled intermediates following turning off the light showed a rapid rise in PGA concentration and a rapid drop in RuDP as well as in fructose and sedoheptulose diphosphate. In an attempt to determine whether one or two molecules of PGA were formed per carboxylation reaction, we studied the rate of increase of PGA after turning off the light following steady state photosynthesis (23). During the first 10 sec after the light was turned off somewhat more than one molecule of PGA appeared per molecule of RuDP which disappeared. This was taken as evidence that in the dark two molecules of PGA were formed per carboxylation of RuDP.

In an effort to learn more about the role of light-produced cofactors in the in vivo carbon reduction reactions, we made a kinetic study of the synthesis of compounds from $^{14}CO_2$ by Chlorella in the dark following preillumination (20). Benson and Calvin (13) had found that the dark fixation of $^{14}CO_2$ by Chlorella is greatly stimulated by preillumination. They found that principal products after 15 sec in the dark following preillumination are PGA, sugar phosphates, malic acid, alanine, and aspartic acid (14).

In our more recent experiments (20), we allowed Chlorella to reach a steady state of photosynthesis with unlabeled carbon. Then, without interrupting the flow of carbon dioxide, we turned off the light and immediately switched from ordinary carbon dioxide to $^{14}CO_2$.

Some of the results of this experiment may be seen in Fig. 2. There was an initial rapid uptake of $^{14}CO_2$ during the first 15 sec. Then there occurred a temporary decrease in $^{14}C$. Presumably some of the newly incorporated $^{14}C$ was respired. After this drop, a slight loss of newly incorporated $^{14}C$ in such products as sugar monophosphate occurs during the period from 15 to 30 sec. Thereafter, $^{14}C$ in these compounds remains essentially constant.

This behavior strongly suggests a mechanism whereby the newly photosynthesized sugar phosphates are prevented from being oxidized by respiratory reactions. From the usual concept of the PSCR cycle in which PGA is reduced by triose phosphate dehydrogenase in the presence of ATP and NADPH, one might have predicted that once these cofactors were exhausted in the dark, the triose phosphate dehydrogenase reaction would be reversed and the newly formed sugar phosphates rapidly oxidized.

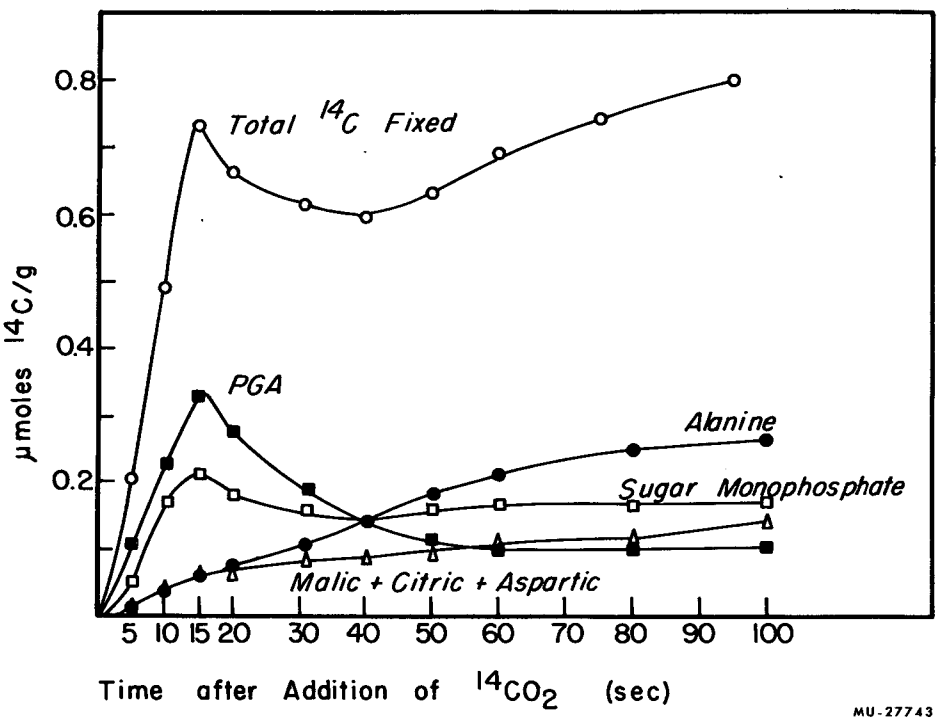


Fig. 2. Fixation of $^{14}C$ into compounds in Chlorella following preillumination. Sugar monophosphates include monophosphates of fructose, glucose, sedoheptulose, ribose and ribulose. "Total $^{14}C$ fixed" includes all radioactive compounds, including those on the origin, found on the chromatogram.

Another very interesting point about these results is the rapid rate of labeling of sugar monophosphates between 5 and 10 sec. This rate is equal in magnitude to the rate of labeling of PGA despite the fact that at that time the PGA carboxyl group can in all probability be no more than 20 to 30% saturated (12). This appears to be the most direct evidence yet for the reduction of some bound form of newly incorporated $^{14}CO_2$ not in equilibrium with the PGA pool.

These results are not, as it might at first appear, inconsistent with the rapid increase in PGA concentration in the dark found earlier (23). In the light-dark steady state experiment following photosynthesis with $^{14}CO_2$, one measures the concentration of the $^{14}C$ saturated pools. In the preillumination experiment intermediates are far from saturated with $^{14}C$, and one sees only the $^{14}C$ labeling of a pool. The size of the pool may be changing in the same or opposite direction as the $^{14}C$ labeling.

The two experiments are consistent if one supposes that when the light is turned off ATP is the first cofactor depleted leading to an immediate onset of the drop in TuDP concentration and

immediate cessation of reduction of the free PGA. One must at the same time suppose that the reduction of some of the bound, newly incorporated $^{14}C$ ("bound PGA") proceeds through the mediation of a stronger reducing agent than NADP, since no ATP is present. This could be a reducing cofactor of the strength of reduced PPNR (chloroplast ferredoxin).

## The Carboxylation Enzyme In Vitro

Carboxydismutase (RuDP carboxylase) converts one molecule of RuDP and one molecule of $CO_2$ to two molecules of 3-PGA (24-26). The enzyme has been prepared from intact chloroplasts by Littleton and Ts'o (27) and by Pon (28). Although many different procedures have been employed for the purification of carboxydismutase (25,28,29), only about 10 to 20 fold increase in specific activity of the enzyme has been achieved (25,28). Ultracentrifugation of purified carboxydismutase has often given two protein components. One of these components with a sedimentation constant of 18 S to 19 S constitutes the major part of the protein (25,28, 29, 27). This fraction contains the carboxydismutase activity (29).

Ultracentrifugation of soluble leaf proteins (30-32) gave a

protein component with an 18 to 19 S sedimentation constant which has been designated "fraction I protein". Fraction I protein has similar physical properties to those of carboxydismutase (30,33) and has carboxydismutase activity (33,25).

Park and Pon (34) showed that lysing of isolated chloroplasts produces protein of which 50% has a sedimentation constant of 16S. This protein and fraction I protein prepared according to the method of Littleton and Ts'o (27) are indistinguishable when observed in the electron microscope. The particles were described as oblate spheroids, 200 Å in diameter. Van Noort (35) suggests that the observed particles may be aggregates of 3 to 5 particles of fraction I protein with an aggregate molecular weight between 1 and 2 million.

Pon (36) found that the activity of the enzyme was increased by preincubation with $HCO_3^-$ and $Mn^{++}$ but not by preincubation with RuDP. Akoyunoglou (37) prepared a complex of enzyme-$^{14}CO_2$. Incubation of this complex with RuDP resulted in the formation of PGA labeled with $^{14}C$.

## Unstable First Products

Moses and Calvin (38) isolated two compounds tentatively identified as "keto acid diphosphates" labeled with $^{14}C$ after about 1 min photosynthesis with $^{14}CO_2$. It appeared that one of these, a compound which was highly unstable, might be an intermediate in the carboxylation reaction. The more stable compound was later (39) shown to be a straight chain keto acid diphosphate unrelated to the expected early product of carboxylation. No kinetics were performed on the formation of the less stable compound that was suspected of being the β-keto acid diphosphate. No further data has appeared to substantiate the suggestion that it possessed the required structure.

Shkolnik and Doman (40) reported several years ago the formation of an unstable $^{14}C$ labeled compound which behaved kinetically like an intermediate in the carboxylation reaction. This compound was isolated by low temperature killing of the plant used and low temperature chromatography. It appeared that it was converted to PGA *in vivo* after a few sec or by thermal reactions if the plant material was killed at room temperature. Further reports on the identity and kinetic behavior of this compound are awaited with great interest.

Galmiche (41) reported an analysis of the products of a few sec photosynthesis with $^{14}CO_2$. In his experiments, the plant was killed in isopentane at -160°C and extracted in the cold with the aid of formamide. The extract was analyzed at 0°C with high voltage electrophoresis. While the identifications are reported to be not yet completed, those compounds which were identified included, in addition to diphosphoglyceric acid and "amino acid phosphates", compounds which have been reported in previous studies with $^{14}CO_2$ fixation during photosynthesis, in which the analysis was performed by two dimensional paper chromatography. Thus, the gentler method of analysis has thus far not revealed any new radioactive compounds that are likely to be unstable first products.

## Inhibition Studies

Kandler and Liesenkotter (42) have reported studies on the fixation of $^{14}CO_2$ during photosynthesis by Chlorella and chloroplasts in the presence of various inhibitors. Studies were also made with labeled glucose in the light and in the dark. They reported that low concentrations ($10^{-5}$ M) of monoiodoacetic

acid (MIAA) did not inhibit glucose respiration or $CO_2$ assimilation by chloroplasts but did inhibit photosynthetic reduction in whole cells. They considered this as evidence that there exists a path of carbon reduction which bypasses PGA. They assumed that this bypass includes the most MIAA sensitive step of photosynthesis.

The principal effect of both arsenate and 2,4-dinitrophenol in their studies was a decrease in the level of ATP and presumably in the level of RuDP.

A basic drawback to most of these experiments seems to be the requirement for a rather long period of preincubation of the cells with the inhibitor. Thus the changes in labeling produced by the inhibitor are somewhat difficult to interpret.

Recently, Kandler and Liesenkotter (43) reported on the effect of KCN on the intermediate concentrations of Chlorella during photosynthesis and during dark respiration. They found that in the dark, besides the inhibition of pyruvate oxidation, glycolysis was inhibited at the aldolase reaction, leading to

an accumulation of fructose diphosphate. During photosynthesis, the carboxylation reaction was the most sensitive to inhibition by KCN and RuDP accumulated. Light phosphorylation was found to be only slightly effected by the inhibitor.

Unlike the Chlorella, broken chloroplasts did not accumulate RuDP with KCN and illumination. In this case, PGA accumulated and sugar phosphates were not formed.

To explain these and earlier observations they proposed that the labeled PGA which appears after a few sec photosynthesis in $^{14}CO_2$ is not free PGA in the cell, but either a labile intermediate such as β keto acid, or PGA bound to an enzyme. They propose that there is a larger pool of free PGA. This larger pool would account for most of the measured PGA concentration in experiments which determine concentration by tracer saturation. They also propose that it is the free pool of PGA which would undergo the large transient changes when the light is turned off or $CO_2$ concentration is lowered.

They suggest that only the bound form of PGA is reduced in whole cells, but that in broken chloroplasts the entire pool of

PGA may be reduced by a reversal of glycolysis. They further suggest that the origin of the free PGA pool lies in glycolysis of labeled sugar phosphates formed by photosynthesis. They suppose the rate of this glycolysis to be accelerated when the light is turned off due to an increased supply of inorganic phosphate. They postulate that $CO_2$ deprivation causes increased binding of phosphate leading to a decrease in inorganic phosphate and an inhibition of glycolysis. This would in turn result in a decrease in the level of the free PGA pool.

In view of the kinetic experiments already mentioned (2,20) the direct reduction of some newly incorporated $CO_2$ to the level of sugar phosphates without equilibrium with the main PGA pool is an attractive hypothesis. Its attractiveness does not depend on acceptance of the idea that the main source of the free pool of PGA is glycolysis of sugar phosphates formed directly from $CO_2$ by photosynthesis. The free PGA pool might be derived from the "bound" PGA.

An explanation of transient effects on PGA pool size based

upon the level of inorganic phosphate is intriguing. It must be noted, however, that we have observed (20) such transient increases in the PGA pool on turning off the light in experiments with Chlorella in which the medium contained 2.0 mμ phosphate. One wonders whether the concentration of phosphate in the chloroplast can become rate limiting under these conditions.

We have recently reported studies with an inhibitor which requires no preincubation (21). When 8-methyl lipoic acid (MLA) is added to a suspension of Chlorella pyrenoidosa which have been photosynthesizing under steady state conditions with $^{14}CO_2$ for a sufficient time to saturate the intermediates of the PSCR cycle, sudden and dramatic changes in the concentrations of some of the intermediate compounds occur. At the same time, carbon dioxide uptake and oxygen evolution are completely inhibited. This inhibition is partially reversed after some 10 to 20 min.

During the first 15 sec after addition of MLA, the concentration of labeled PGA drops at a rate comparable to the rate of uptake of $^{14}CO_2$ from the external medium. At the same time, the concentrations of $^{14}C$ labeled fructose-1,6-

diphosphate and sedoheptulose-1,7-diphosphate rise very rapidly, while the concentration of their respective monophosphates decrease appreciably. Surprisingly the concentration of RuDP undergoes only relatively small changes and levels off at a value slightly less than the steady state photosynthesis concentration.

Following the initial transient changes there is an acceleration of the formation of labeled sucrose which climbs to a very high value and then drops rapidly during the period of recovery from inhibition.

The inhibition of oxygen evolution and $CO_2$ uptake by MLA could be explained in terms of an interference of the inhibitor with electron transport from PPNR (chloroplast ferredoxin) to the carbon cycle. If so, it is very interesting to note that the concentration of PGA drops rapidly, whereas interference with its reduction would tend to cause its concentration to increase.

The relatively small response of the concentration of labeled RuDP on addition of MLA compared to the great change in the level of PGA is noteworthy. What appears to be an inhibition of the conversion of fructose and sedoheptulose diphosphates to their respective monophosphates must also be explained, perhaps in terms of a phos-

phatase inhibition. It is premature to speculate on the basis of the preliminary report of this work. It does appear that use of this inhibitor, as well as lipoic acid itself, which also causes inhibition, affords a promising approach for learning more about the operation of the carbon reduction cycle in vivo.

## Glycolic Acid

The formation of labeled glycolic acid can perhaps be explained as an oxidation of glycolaldehyde thiamine pyrophosphate addition compound, formed during the transketolase reactions of the cycle (6). We have previously reported that oxygen stimulates the formation of labeled glycolic acid during photosynthesis with $^{14}CO_2$ (44) (See Fig. 1). The enzymic oxidation of such a compound, called more precisely (2-(1,2-dihydroxyethyl)-thiamine pyrophosphate, by artificial electron acceptors has been demonstrated (46).

The photosynthetic pathway to glycolic acid may be reversible in the light, presumably by activation of glycolic acid with ATP (see Fig. 1). Administered $^{14}C$ labeled glycolic acid is converted to α and β carbons of PGA by photosynthesizing plants. This conversion is accompanied by randomization of label between the

two carbon atoms (47). Presumably a symmetrical intermediate compound is involved.

Formation of $^{14}C$-labeled glycolic acid in photosynthesis is also favored by low $CO_2$ pressure (17,47-50). In this case it may result in part from oxidation of RuDP (which increased greatly in concentration at low $CO_2$ pressure) to phosphoglycolic acid. This compound in turn could be hydrolyzed by glycolic acid phosphatase (51).

## An Organized Enzyme System?

I have suggested elsewhere (45,20) that the carbon reduction cycle of photosynthesis might be mediated by an organized system of multifunctional enzymes. Specific examples were given of the kind of mechanisms which might be involved. These mechanisms, which utilize enzyme bound disulfide and thiazolium groups, were illustrative examples, and their correctness is not essential to the basic concept of the organized multifunctional system.

An essential feature of the proposal was the existence of

a direct link between the photoelectron transport system and the carbon reduction cycle system. This link, which is presumed to be disrupted during chloroplast isolation procedures, would provide for the direct utilization of the high energy reducing cofactors, such as reduced chloroplast ferredoxin, by the enzymes of the carbon reduction cycle. Also conceivable, but not essential, would be a direct utilization of high energy phosphate precursors of ATP, rather than ATP itself.

While the existence of an organized enzyme system for carbon reduction cannot be proved at this time, there are a number of indications that support this hypothesis.

1. Direct evidence for the existence of multifunctional enzyme systems would be the characterization of morphological units of appropriate size and properties. In his review of the biosynthesis of fatty acids, Lynen (52) has discussed properties of a multifunctional enzyme system for the synthesis of fatty acids from malonyl coenzyme A. The purified fatty acid synthetase has a calculated molecualr weight of about 2 million, and could be seen by electron microscopy to be homogeneous particles with visible substructure.

As already mentioned, Park and Pon, using electron microscopy

to view protein obtained by lysing chloroplasts, observed particles 200 Å in diameter (35) which may be aggregates of 3 to 5 fraction I protein molecules as usually isolated by ultracentrifugation (34). This is not conclusive evidence that the larger particles occur *in vivo*, as they could be formed by an artifactual aggregation of fraction I protein. However, the larger particles are of uniform size, so even if they are aggregates the aggregation might be a reversal of a previous dissociation of naturally occurring large particles to fraction I protein. A better knowledge of the morphology of the naturally occurring system may have to await improved techniques for viewing the system *in situ*.

In any event, there must be important differences between the kind of system responsible for fatty acid synthesis and that required for the PSCR cycle. The latter biosynthetic pathway requires more steps and is known to include a number of measurable free pools of intermediate compounds. The PSCR cycle could be mediated by an organized system of 2 to 4 multifunctional enzymes, each no larger than fraction I particles. These particles could be organized along a membrane surface or within membrane-enclosed compartments. The

mere isolation of the major fraction of soluble protein from chloroplasts as a relatively large and homogeneous species is indicative of some such system.

2. The high rate of labeling of sugar phosphates during the preillumination experiment (20) discussed in the previous section suggests the reduction of newly incorporated $^{14}CO_2$ without its equilibration with the free pool of PGA. As discussed earlier, this possibility is further emphasized by the finding in other experiments (23) that PGA concentration is rapidly rising at the same time, suggesting that reduction of free PGA is already blocked almost immediately when the light is turned off.

The extrapolation to zero time of fixation of percentage $^{14}C$ incorporated in various compounds during photosynthesis with $^{14}CO_2$ showed a 10 to 15% incorporation into sugar phosphates. Again, this suggests the reduction of some bound form of $^{14}C$-PGA.

3. The apparent block on the reoxidation of labeled sugar phosphates during the period after 30 sec in the preillumination experiments (20) was discussed in the previous section. Such a block

would require some mechanism beyond that which would be available if the interconversion between PGA and sugar phosphates were accomplished only by triose phosphate dehydrogenase and the cofactors ATP/ADP and NADPH/$NADP^+$. Such a block can be readily visualized if one supposes the carboxylation reaction to lead to a bound form of glyceraldehyde phosphate which is not rapidly oxidized *in vivo* (45).

4. The absence of measurable amounts of 3-phosphoglyceraldehyde and of 4-phosphoerythrose, in contrast to the measurable amounts of all the other postulated intermediates in the cycle, suggests that these moieties may be bound to the enzyme. Perhaps they are bound by enzyme sulfhydryl groups, as HS-Enz-S-CO-R.

5. The fact that no one has been able to isolate and identify an intermediate in the carboxylation reaction to date must be considered as suggestive evidence for the existence of enzyme-bound intermediates through at least two stages of the carboxylation reaction.

6. The inadequacies and variabilities in the activities of isolated enzymes which would catalyze the various reactions of the carbon cycle might be best explained in terms of a multifunctional

enzyme system. Such a large and easily disaggregated enzyme system would be very difficult to isolate intact. It is known that the organization of the lamellae and stroma systems of chloroplasts varies considerably from one organism to another. So also do the techniques required to break cell walls and separate soluble or particulate fractions from intact cells. It would not be surprising therefore if the amounts or presence of specific enzyme activities isolated from such a multifunctional organized system would vary greatly from one organism to another.

Also, the secondary biosynthetic reactions occurring in chloroplasts will vary greatly from one organism to another. Given a multifunctional enzyme system for the basic carbon reduction cycle, an organism in some cases may not require a specific fructose diphosphate aldolase, as assayed by conventional methods. Thus, the absence of such an enzyme in specific organisms which has been reported by Richter (53) and by Fewson et al.

(54,55) may mean merely that that organism had no need to form PGA in the chloroplast by oxidation of fructose-1,6-diphosphate. Conceivably such organisms make all the PGA they need by liberation of "bound PGA" formed in the primary carboxylation reaction.

7. The C-3 and C-4 difference part of the Gibbs effect has been explained earlier in terms of a small pool of glyceraldehyde-3-phosphate and a large pool of dihydroxyacetone phosphate. Such a difference in pool size would be readily explained in terms of the multifunctional enzyme system, if the glyceraldehyde-3-phosphate moiety is enzyme bound. The other part of the Gibbs effect, discrepancy between carbons 1 and 2 as compared with 5 and 6, could also readily be explained. In this case, one would suppose that the enzyme binds a common pool of C-1 and C-2 from transketolase reactions on fructose-6-phosphate, sedoheptulose-7-phosphate and xylulose-5-phosphate. Thus one could account for the reflection of pentose C-1 and C-2 labeling back into hexose C-1 and C-2 labeling. (See Fig. 1.)

8. The kinetic and thermodynamic advantages of such a

multifunctional organized enzyme system for biosynthetic pathways 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 the carbon reduction cycle during photosynthesis, one is led to suspect the existence of such a system for this process. Its basic importance as a 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.

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