DYNAMICS OF THE PHOTOSYNTHESIS OF CARBON COMPOUNDS

I. CARBOXYLATION REACTIONS

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SUMMARY

Kinetic studies have been made of the rates of appearance of ^{14}C in individual compounds formed by *Chlorella pyrenoidosa* during steady state photosynthesis with $^{14}CO_2$. These rates have been compared with rates of CO_2 and ^{14}C disappearance from the gas phase during the same experiments.

The following results were obtained:

1. After the first few seconds, the rate of appearance of ^{14}C in compounds stable to drying on planchets at room temperature is 95 to 100 % of the rate of uptake of carbon from the gas phase.

2. After the first few seconds, the rate of appearance of carbon in compounds isolable by usual methods of paper chromatography constitutes at least 73 to 88 % of the rate of uptake of carbon from the gas phase. Compounds formed from the carbon reduction cycle via the carboxylation of ribulose diphosphate account for a least 70 to 85 % of the uptake, while carboxylation of phosphoenolpyruvic acid appears to account for at least another 3 %.

3. The induction period in the appearance of ^{14}C in stable compounds may be due to a reservoir of intracellular CO_2 and HCO_3^- or to some other volatile or unstable compound. If so, this reservoir contains no more than 1.5 μmoles of carbon, corresponding to about 7 sec carbon fixation in the experiment in which it was measured.

4. No other carboxylation reactions, such as the carboxylation of γ-aminobutyric acid, could be observed. The rate of labeling of glutamic acid after 5 min of exposure of the algae to $^{14}CO_2$ reached a maximum rate of about 5 % of the total uptake rate, but this labeling appears to be due to conversion of labeled intermediates formed from the carbon reduction cycle or phosphoenolpyruvic acid carboxylation.

5. The *in vivo* carboxylation of ribulose diphosphate in the light appears to be followed by conversion of the product to one molecule of phosphoglyceric acid, containing the newly incorporated $^{14}CO_2$ and one molecule of some other (kinetically distinguishable) three carbon compound. This reaction would be different from the one reported for the isolated enzyme system and the *in vivo* reaction in the dark, which produces two molecules of 3-phosphoglyceric acid.

Abbreviations: PGA or 3-PGA, 3-phosphoglyceric acid; PEPA, phosphoenolpyruvic acid; RuDP, ribulose 1,5-diphosphate; ATP, adenosine triphosphate; TPNH, reduced triphosphopyridine nucleotide.

INTRODUCTION

Much of the biochemical pathway through which carbon dioxide is reduced during photosynthesis in algae has been established[1-3]. A principal feature of this pathway is the carbon reduction cycle. A simplified version of this cycle is given in Fig. 1, which shows the key steps.

To map these paths, CALVIN *et al.*[3-10] gave radioactive compounds, such as $^{14}CO_2$ and $KH_2{}^{32}PO_4$, to photosynthesizing plants. The plants made various reduced organic compounds from these labeled substrates. They were then killed and the soluble compounds were extracted from the plant material and analyzed by two-dimensional paper chromatography and radioautography. The compounds were identified and their radioactive content determined. From the amount and location of radioactive elements within compounds following exposures of the plants for various lengths of time and under various environmental conditions, biochemical pathways were followed.

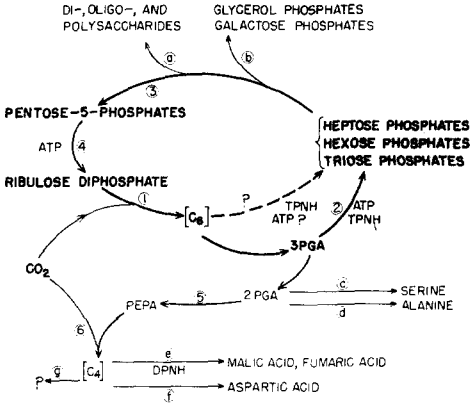


Fig. 1. Carbon reduction cycle (simplified version). (1) Ribulose diphosphate reacts with CO_2 to give an unstable six carbon compound which splits to give two three carbon compounds. At least one of these is 3-phosphoglyceric acid. The other three carbon compounds might be either 3-PGA, as it is known to be in the isolated enzyme system, or some other three carbon compound such as a triose phosphate (dashed arrow). (2) PGA is reduced to triose phosphate with ATP and TPNH derived from the light reaction and water. (3) Various condensations and rearrangements convert the triose phosphates to pentose phosphates. (4) Pentose phosphate is phosphorylated withATP to give ribulose diphosphate. Further carbon reduction occurs *via* conversion of PGA to phosphoenolpyruvic acid, (5), and carboxylation, (6), to form a four carbon compound (probably oxaloacetic acid). Reactions leading to the formations of some of the secondary intermediates in carbon reduction are shown by the arrows lettered a through g.

In the present study we have extended our information about these pathways by more precise control of the environmental conditions during exposure of the plants to tracers. At the same time we have made measurements of the rate of entry of tracer into the plant and of the rate of appearance of the tracer in specific compounds.

We sought answers to the following questions: (a) How much of the total carbon taken up by the plants enters the metabolic network via carboxylation of ribulose diphosphate (reaction 1)? (b) How much of the total carbon taken up enters by carboxylation of PEPA (reaction 6)? (c) Are any other carboxylation reactions, such as the carboxylation of γ-aminobutyric acid[11], of any importance in steady state photosynthesis? (d) Does the carboxylation of ribulose diphosphate *in vivo* lead to one product only (PGA) or does it lead to two products (PGA and some other 3-carbon compound)?

"Steady state photosynthesis" as used in this paper, is defined as a condition under which unicellular algae are carrying out the reaction of photosynthesis, are synthesizing all of the normal cell constituents, and are growing and dividing at

constant rates during the course of the experiment. Moreover, the rates of photosynthesis in experiments which will be reported here were between 30 and 80 % of the maximum rates at which these algae are capable of photosynthesizing at room temperature.

EXPERIMENTAL

Plant material

The plants used in all experiments were the unicellular green algae, *Chlorella pyrenoidosa*, raised in continuous automatic culture tubes as described previously[3]. The algae were raised and harvested as a 0.5 % (volume wet packed cells/volume) suspension. The algae were centrifuged from the culture medium and then suspended in a special nutrient solution (described later). This suspension (80 ml) was placed in the illumination chamber of the steady state apparatus.

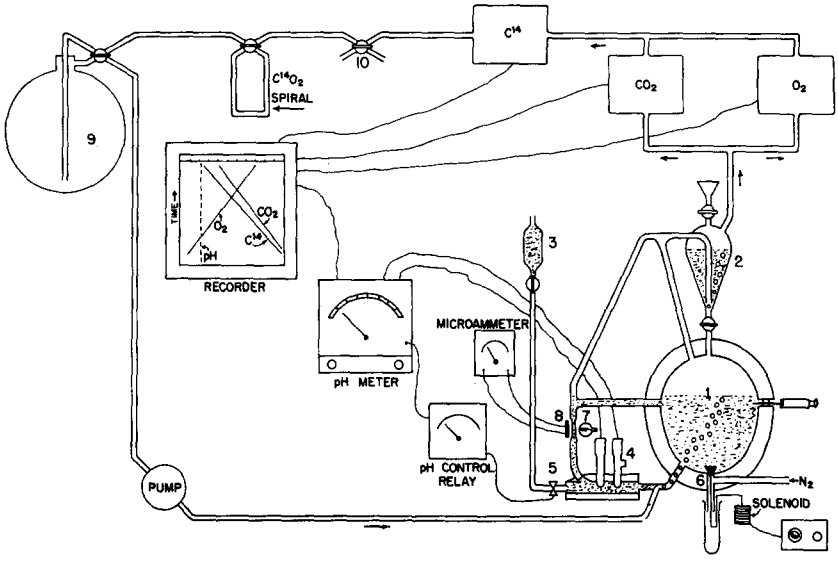


Fig. 2. Steady state apparatus. (1) algae chamber, (2) water or nutrient solution reservoir, (3) acid or base reservoir, (4) pH electrodes, (5) solenoid operated pH control valve, (6) solenoid operated sampling valve, (7) small lamp, (8) photovoltaic cell, (9) large gas reservoir, (10) four-way stopcock.

Steady state apparatus

In the steady state apparatus, shown schematically in Fig. 2, a stream of gas (1 to 2 % CO_2 in air) is cycled through a closed system. The gas is bubbled through the 0.5 % or 1.0 % suspension of algae (80 ml) at a rate of approximately 1 l/min. Gas and liquid mix rapidly in the algae chamber, which is 3/8″ thick and 4″ in diameter (inside dimensions). The algae chamber is illuminated from both sides by G.E. RSP2 photospot incandescent lights through an infrared absorbing glass in a water bath, or in some experiments from one side by an incandescent lamp and from the other side by a bank of eight 8″, 6 W fluorescent lamps (blue and cool white). In either case, the voltage to the incandescent lamps is adjusted just to give light saturation of the oxygen evolution rate. The algae chamber is water jacketed, and

the water is circulated in a thermostated bath. The temperature of this bath is set so that during steady state photosynthesis the temperature indicated by the thermometer in the algae suspension reads 25°.

The algae chamber is connected to a side loop through which the algae suspension is made to circulate by the flow of gas into the chamber. A beam from a small lamp passes through a window in the side loop to a photovoltaic cell which measures the light absorption and hence the density of the algae. Electrodes in the side loop measure pH, which is recorded on a multipoint recorder. The pH meter output is also connected to a control relay which, through the activation of a solenoid-operated valve, can cause acid or base from a reservoir to be added in small volumes to the algal suspension. Another reservoir within the closed system contains distilled water or nutrient solution, which can be added to the algal suspension to dilute it to the selected concentration as the algae grow.

A solenoid-operated sampling valve at the bottom of the chamber permits one to take 1-ml samples rapidly (every 2 sec if desired). The inside of the algal chamber is maintained at slightly above atmospheric pressure to force the algal sample out of the chamber. When samples of algae are taken, they are run into 4 ml of methanol at room temperature. This gives a mixture which contains about 80% methanol by volume. No significant difference in the resulting labeling pattern is seen whether the algae are killed this way, in boiling ethanol, or in ethanol kept at —40°.

After the gas in the closed system bubbles through the algae, it passes through instruments which measure CO_2, ^{14}C, and O_2, and each measurement is automatically recorded. From the known sensitivities of these instruments and the volume of the system, one can calculate rates of exchange of these quantities and specific radioactivity. A large reservoir and small reservoirs may be connected or disconnected from the closed system to obtain closed systems of various sizes. The volume of the largest system is 6400 ml, while the volume of the smallest system is 435 ml. The system can be open during the pre-labeling period by means of a stopcock.

Nutrient solution

For steady state experiments it is necessary to supply the algae with all the inorganic compounds required for them to photosynthesize and grow at a normal rate. Unfortunately, the nutrient solution in which they are usually grown in the laboratory contains quantities of salts which make impossible an adequate separation of labeled compounds by two-dimensional paper chromatography. Therefore, the algae are suspended in much more dilute nutrient solutions of which that in Table I is typical.

TABLE I

STARTING NUTRIENT SOLUTION FOR STEADY STATE EXPTS. 18 AND 28

$(NH_4)_2HPO_4$	40 mg/l
$MgSO_4 \cdot 7H_2O$	20 mg/l
NH_4Cl	20 mg/l
KNO_3	20 mg/l
ARNON's A-4 solution of trace elements plus $CoCl_2 \cdot 6H_2O$ (40 mg/l) and MoO_3 (15 mg/l)[12]	1 ml/l
Fe^{++}-versenol solution to give 90 mM Fe^{++}	1 ml/l
NH_4VO_3 (23 mg/l)	1 ml/l

This medium was adequate to maintain nearly a constant rate of photosynthesis in experiment steady state No. 18. In other experiments, such as steady state 28, the algae growing under steady state conditions would in time exhaust the supply of ammonium ion contained in this medium. However, it has been observed that as the algae take up ammonium ion, the pH of the medium tends to decrease, presumably due to the exchange into the medium of hydrogen ions for ammonium ions. Therefore, dilute NH_4OH was added to the algae suspension automatically by the pH control system, thereby maintaining constant pH. At the same time ammonium ion concentration was maintained approximately constant. The nutrient solution for pH control was diluted by trial and error until its addition kept the algae density constant. To it were added other inorganic ions in a ratio to the ammonium ion which was estimated to provide the algae with an adequate level of these ions for growth for a limited period. The resulting pH control medium used in steady state experiment 28 is shown in Table II.

TABLE II

CONTROL MEDIUM USED IN STEADY STATE EXPERIMENT 28

$(NH_4)HPO_4$	6.6 mg/l
$(NH_4)_2SO_4$	6.6 mg/l
NH_4OH	0.55 mg/l
$FeCl_3 \cdot 6H_2O$	5.0 mg/l
KCl	8.0 mg/l

Trace elements as in starting medium

Administration of ^{14}C

During the first part of the experiment the algae are kept photosynthesizing in the light with a constant supply of 1.5 to 2 % unlabeled CO_2 in air for 0.5–1 h. Constant pH, temperature, and light intensity are maintained during this time, and during the subsequent exposure to $^{14}CO_2$. In the experiments reported here the pH was kept at 6. Rate measurements of CO_2 uptake and O_2 evolution are made by making the closed system small, 435 ml for a few minutes, and observing the rate of change of CO_2 and O_2 tensions as indicated on the recorder. The closed gas system is made large again, and at zero time, $^{14}CO_2$ is added to the system by turning a stopcock. At the same instant a solution of $NaH^{14}CO_3$ is injected directly into the algal suspension. The amount and specific radioactivity of the injected bicarbonate solution is so calculated that it will immediately bring the specific radioactivity of the dissolved CO_2 and bicarbonate already present in the algal suspension to its final value. This is the specific radioactivity which will obtain for all the CO_2 and bicarbonate in the gas and liquid phases of the closed system after complete equilibration has occurred. An example of this calculation is given in Table III. Samples of the algae suspension of uniform size are taken every 5 or 10 sec for the first few minutes, and then less frequently for periods up to 1 h. Each sample is taken directly into 4.0 ml of methanol (room temperature) in a centrifuge tube (preweighed). Sample tubes are reweighed to give the sample size ($\pm$ 1 %). After an hour at room temperature, the samples are centrifuged and the 80 % methanol extract removed. 1 ml of methanol is added to the residue and stirred a few minutes, then 4 ml water is added and the mixture

TABLE III

CALCULATION OF $^{12}C + ^{14}C$ FOR STEADY STATE EXPT. 18

	Volume	*%CO_2*	*μmoles*	*μ$C^{14}C$*	*Specific activity*
A Gas phase at start	895	1.6	585	0	0
B $^{14}CO_2$ loop	72		156	3767	
C Dissolved CO_2, HCO_3	125*		81.6		0
D $NaH^{14}CO_3$ injected			40.8	607.5	
Total	1092		863	4375	5.07 μC/μmole
C + D			122.4	607.5	4.95 μC/μmole

* Effective volume.

warmed at 60° for 10 min. After centrifugation and a further extraction with 1 ml of water, the combined clear extracts are concentrated at reduced pressure at below room temperature. The concentrated extract, or an aliquot portion thereof, is transferred quantitatively to the paper chromatogram and analyzed in two dimensions (phenol–water, butanol–propionic acid–water) as in earlier work[5]. The location of the radioactive compounds on the chromatograph is found by radioautography with X-ray film. When necessary, overlapping phosphate esters are eluted, treated with phosphatase and rechromatographed.

Determination of radioactivity in compounds

The amounts of radiocarbon in each compound of interest on the chromatograms from each sample is measured with a Geiger-Mueller tube. The paper chromatogram is placed on top of the radioautograph, which rests on a horizontal light table, so that the darkened areas of the film may be seen through the paper. The Geiger-Mueller tube has a Mylar window, gold-sputtered for conductivity, but transparent and thin (less than 1 mg/cm^2) to permit the passage of ^{14}C beta particles. This tube has an effective counting area of uniform sensitivity of about 17 cm^2. The top of the tube is transparent plastic so that paper and radioautograph may be viewed through the top of the tube. Thus the counting area of the tube may readily be placed in position over the radioactive compound on the paper. If the radioactive area is more than 4 cm across, or if it contains more than 20,000 counts/min (as counted by this tube on the paper), the radioactive area is divided into smaller areas which are counted one at a time (with the remainder of the spot covered by cards). The counting gas used is helium–isobutane (99:1). The counting voltage is about 1300 V. The sensitivity of the counter for ^{14}C beta particles in an infinitely thin layer on an aluminum planchet is about one count/3.1 disintegrations. However, only about one-third of the beta particles escape from the paper (Whatman No. 4) and the actual sensitivity of this tube for ^{14}C in compounds on the paper is about 1/11.2. These sensitivities were determined by comparison of counts from three aliquot portions of a known ^{14}C labeled solution: (a) chromatographed on paper, (b) dried on a planchet, and (c) placed in a scintillation counter with an internal standard. The radioactivity of each compound is counted on each side of the paper and an average is taken of the counts from the two sides. Comparison with determinations of radioactivity of compounds quantitatively eluted and placed on planchets indicates that this method of counting gives an accuracy of ± 5 %.

Rate measurements

Gas exchange: Measurements of the rates of CO_2 uptake, ^{14}C uptake and O_2 evolution by the photosynthesizing algae are made by taking the slopes of the three traces on the recorder. In order to obtain accurate readings in 10 min or less, the total effective gas volume of the closed circulating system is made small, about 435 ml. With 80 ml of 0.5 % algal suspension in the system the resulting change in O_2 or CO_2 pressure is about 0.5 % in 10 min in a typical experiment. This corresponds to a rate of 22 μmoles of gas exchange/min/ml of wet packed algae. The response of the Beckman Infrared Analyzer, model 15 A, used in these experiments is not completely linear in the range used (0 to 2.0 % CO_2) so that a correction based on a previously obtained calibration curve is applied to the CO_2 uptake curve plotted on the recorder. The response of the A. O. Beckman oxygen analyzer is essentially linear in the range used (19 to 21 %). The level of ^{14}C is plotted on the recorder as millivolts response of the Applied Physics Corpn.'s Vibrating Reed Electrometer to the ionization chamber (volume 118 ml, R = 10^9 ohms). From the known calibration of the ionization chamber this reading can be directly converted to μC of ^{14}C. From the $^{12}CO_2$ reading and the ^{14}C reading the specific radioactivity of the CO_2 may be calculated at all times during the experiment. This specific radioactivity is used to convert the rates of change of radioactivity in the system to rates of change of what we shall call "^{14}C" throughout this paper. For convenience of expression and calculation, this ^{14}C will be expressed in μmoles and represents the amount of ^{12}C and ^{14}C corresponding to a given measured amount of radioactivity in the CO_2 administered to the algae at any time during the experiment.

Total fixation in algae: In some experiments, small aliquot portions of each sample of algal material, taken and killed in alcohol during the course of the experiment, are spread in a thin layer on planchets with acetic acid, dried, and counted. The amount of ^{14}C found at each time of exposure of the algae to $^{14}CO_2$ is plotted and the slope of the curve drawn through these points gives the rate of appearance of ^{14}C in stable compounds in the plant.

Fixation of ^{14}C in compounds found on the paper chromatograph: After the ^{14}C in individual compounds found on the paper chromatogram has been measured, the amounts are sometimes totaled for each sample up to one minute, and a rate of appearance of ^{14}C in these compounds is calculated.

RESULTS

Steady state Expt. 18

The rates of exchange of gases before, during and at the end of the experiment are shown in Table IV. We shall take 15.5 μmoles/min as an average value for uptake of carbon during the experiment.

Aliquot portions of the samples were dried on planchets and their radioactivity was counted. When results of these counts were plotted *versus* time of sampling, the rate of fixation of ^{14}C into compounds stable to drying on the planchets was found to be about 15 μmoles/min (Fig. 3).

After chromatographic separation of the compounds, radioautographs, of which Fig. 4 is typical, were obtained. The radioactivity of each compound in each sample was determined and the total radiocarbon found in the various compounds

TABLE IV

RATES OF GAS EXCHANGE IN STEADY STATE EXPT. 18

All rates are given in μmoles/ml of wet packed algae

	Carbon dioxide	^{14}C*
Initial rate	17.9	
During experiment	16.6	15.1
Final rate	14.1	13.7

* See section *Methods of measurements of rate of gas exchange* for explanation of expression of ^{14}C in μmoles. In theory the value for ^{14}C and CO_2 should be the same. The difference is a reflection of inaccuracy in measurement of the slopes, especially CO_2.

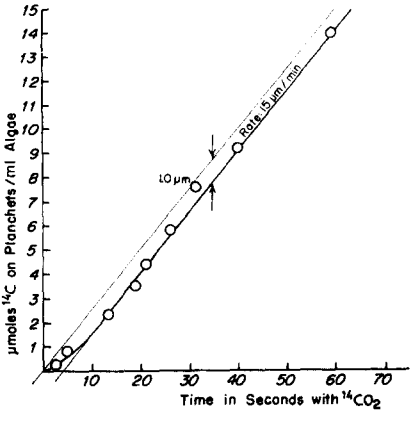


Fig. 3. Appearance of ^{14}C in stable compounds (dried on planchets) in *Chlorella pyrenoidosa vs.* time of photosynthesis with $^{14}CO_2$.

Fig. 4. Radioautograph of chromatogram of *Chlorella pyrenoidosa* after 2 min photosynthesis with $^{14}CO_2$.

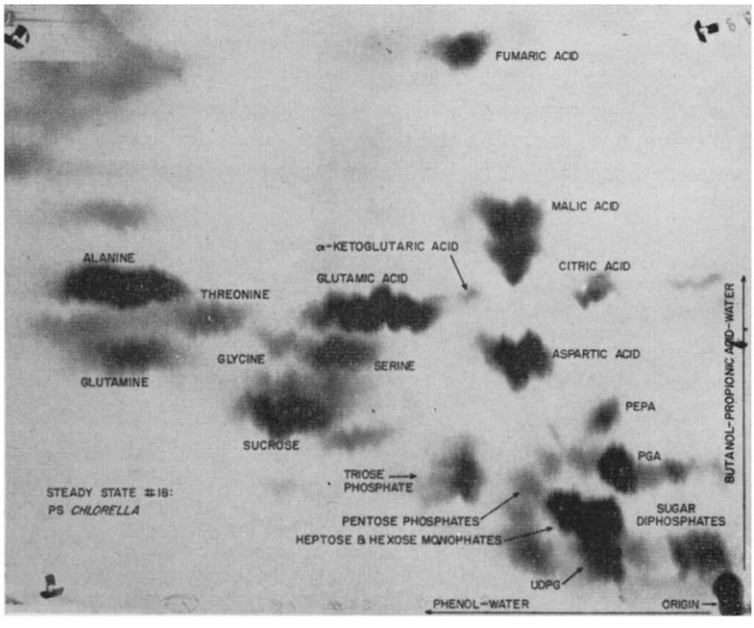


Fig. 4.

was plotted against time (Fig. 5). The maximum slope of the curve in Fig. 5 is 13 μmoles. This is a lower limit for the rate of appearance of ^{14}C in soluble compounds which are also stable to chromatography. It does not take into account other compounds, too weakly radioactive to be counted, or "lost" from the front of our chromatograms. (In order to obtain good separation of phosphate esters we customarily allow the phenol–water solvent to drip from the ends of the chromatograms. Small amounts of labeled fatty material are lost in this way.)

After 30 sec, appreciable amounts of radioactivity are passing through the extractable precursor compounds seen on the chromatograms into non-extractable substances, which are not seen on the chromatograms. Consequently the rate of appearance of ^{14}C in compounds on the paper decrease.

During the first ten seconds, the rate of appearance of ^{14}C in stable compounds is less than the maximum rate during the subsequent time. This could be ascribed to mixing time of the added $H^{14}CO_3^-$ with the $H^{12}CO_3^-$ present initially, or alternately to the presence of an intermediate pool of either HCO_3^- or some other unstable or

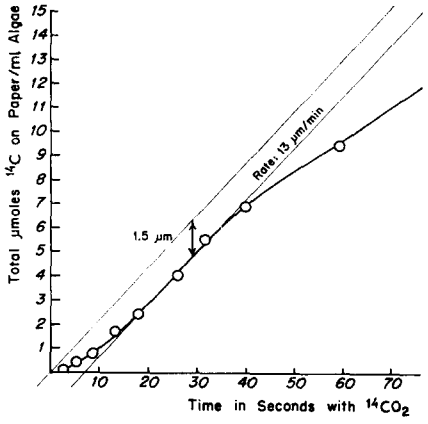


Fig. 5. Appearance of ^{14}C in compounds on chromatograms prepared from *Chlorella pyrenoidosa vs.* time of photosynthesis with $^{14}CO_2$.

volatile compound. Such a compound would precede the stable soluble compounds in the fixation pathway. The size of this "pool", if it exists, cannot be greater than the difference between the fixation curve after 10 sec and a line of the maximum slope drawn through the origin (see Figs. 3 and 5). This is no more than 1.0 to 1.5 μmoles, which is equal to the carbon fixed in 4 to 6 sec in this experiment. A calculation of the amount of HCO_3^- which would be found inside algae cells in a volume of 1 ml with an internal pH of 7 in equilibrium with 1.7 % CO_2 gives a value of about 1 to 1.5 μmoles, depending on the volume available inside the cells. It seems to us to be not unreasonable to suppose that this "pool" is merely intracellular CO_2 and HCO_3^- but it does not matter to the subsequent argument whether it is this or some other unstable or volatile substance.

From the measured rates of uptake of CO_2 and ^{14}C and from the rates of appearance of ^{14}C in stable compounds these experimental findings may be listed: (a) The appearance of ^{14}C in stable, nonvolatile compounds, after the first 10 sec of exposure of the plant to $^{14}CO_2$, is equal to the rate of total uptake of $^{14}CO_2$ within

experimental error. (b) During the period between 10 and 30 sec exposure to $^{14}CO_2$, the appearance of ^{14}C in individual compounds which can be isolated by our methods of paper chromatography, is equal to at least 85 % of the rate of total uptake. (c) If there is a pool of CO_2, HCO_3^- or other unstable or volatile compound lying between administered CO_2 and stable compounds in the fixation pathway, its amount is not more than 1.0 to 1.5 μmoles (4 to 6 sec fixation) and it is essentially saturated after 10 sec.

Let us next consider the question of how much of this fixed ^{14}C must pass through the PGA pool.

In Fig. 6 are shown the labeling curves of some of the more rapidly labeled compounds and groups of compounds. By 3 min, compounds of the carbon reduction cycle are essentially saturated with radiocarbon. Secondary intermediates such as sucrose, malic acid, and several amino acids are not saturated until longer times (5 to 30 min). In order to evaluate the importance of the fixation pathway leading through PGA, we have tabulated the actual measurements of ^{14}C found in compounds

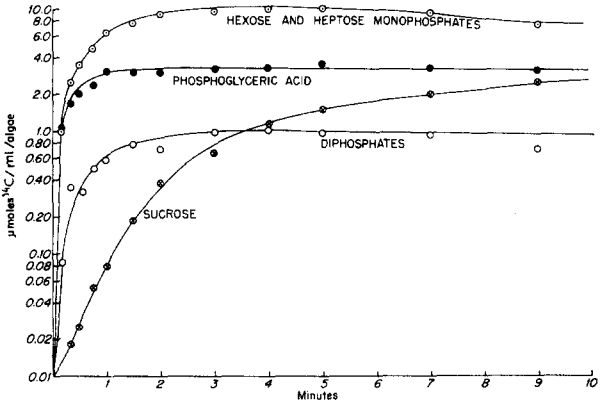


Fig. 6. Appearance of ^{14}C in PGA and sugar phosphates in *Chlorella pyrenoidosa vs.* time of photosynthesis with $^{14}CO_2$.

during the first minute (Table V). The ^{14}C found in all those compounds derived from PGA without further carboxylation (see Fig. 1) is added together (T_1). Compounds labeled by C_3–C_1 carboxylation are totaled separately (T_2). Since three of the carbon atoms in these compounds are derived from PGA, their total radioactivity is multiplied by a factor which is 3/4 times the degree of saturation of the PGA, which is presumed to be the same as that of their immediate precursor, namely, PEPA. (The saturation curves for PGA and PEPA are in fact very similar in this and other experiments.) The sum of T_1 and T_2f, representing measured ^{14}C derived from the primary reaction which forms PGA, is plotted in Fig. 7. Again the "pool" of HCO_3^- or other volatile or unstable compound is about 1 μmole and in this case it must precede PGA in the chain of reactions. Where one draws the curve of maximum slope through these points is somewhat arbitrary, but the maximum rate of appearance of ^{14}C in these compounds falls somewhere between 11 and 13 μmoles/min. Thus on the basis of the appearance of ^{14}C in these extractable, stable compounds alone, at least 70 to 85 % of all carbon fixed during photosynthesis (measured externally) is incorporated via the carbon reduction cycle. It must be emphasized

TABLE V

^{14}C (μMOLES/ML ALGAE) IN PHOTOSYNTHESIS INTERMEDIATES DURING FIRST MINUTE WITH $^{14}CO_2$

Steady State *Chlorella* 18.

Compound	*Time in seconds of $^{14}CO_2$*									*Steady state value*	
	2.5	*5*	*8.5*	*13*	*18*	*26*	*31.5*	*40*	*59*	*µmoles carbon*	*µmoles compound*
PGA	0.063	0.220	0.466	0.750	0.983	1.38	1.72	1.92	2.29	3.0	1.0
PEPA	0.0084	0.0083	0.026	0.041	0.047	0.093	0.114	0.132	0.170	0.2	0.07
Hexose monophosphate	0.017	0.063	0.205	0.429	0.677	1.14	1.60	1.99	2.73	5.5	0.9
Heptose monophosphate	0.011	0.027	0.080	0.157	0.247	0.406	0.617	0.766	0.955	1.8	0.26
Pentose monophosphate	0.0021	0.0096	0.011	0.024	0.035	0.054	0.074	0.067	0.133	$\sim$ 0.2	$\sim$ 0.04
Triose monophosphate	0.0069	0.0139	0.038	0.063	0.084	0.144	0.174	0.215	0.283	$\sim$ 0.8	$\sim$ 0.3
Diphosphates except ribulose	0.0041	0.013	0.026	0.044	0.053	0.093	0.138	0.160	0.203	$\sim$ 0.54	—
Ribulose diphosphate	0.0045	0.0129	0.028	0.047	0.057	0.100	0.150	0.190	0.225	0.36	0.07
Alanine	—	0.025	0.024	0.049	0.075	0.170	0.257	0.397	0.869	$\sim$ 15	$\sim$ 5
Serine	—	0.015	0.012	0.016	0.013	0.027	0.039	0.053	0.120	1.2	$\sim$ 0.4
Sucrose	—	—	—	—	0.0080	0.019	0.021	0.035	0.094	$>$ 3.5	$>$ 0.3
UDPG	—	—	—	0.0090	0.019	0.048	0.108	0.170	0.308	$>$ 2.0	—
Total (T_1)	0.117	0.407	0.916	1.629	2.298	3.674	5.012	6.085	8.380		
Aspartic acid	—	—	0.0070	0.015	0.025	0.063	0.091	0.131	0.299	2.0	0.5
Fumaric acid	—	—	0.0020	0.0030	0.0080	0.013	0.025	0.050	0.091	0.7	0.18
Malic acid	—	0.0066	0.018	0.040	0.063	0.152	0.194	0.271	0.480	2.1	0.5
Total (T_2)		0.007	0.027	0.058	0.096	0.228	0.310	0.452	0.870		
x = PGA saturation	0.021	0.073	0.152	0.250	0.327	0.461	0.575	0.635	0.765		
f = 3x/4	0.016	0.055	0.116	0.158	0.246	0.347	0.432	0.476	0.574		
$T_2 \times f$	—	—	0.002	0.009	0.023	0.078	0.135	0.216	0.500		
Grand total = $T_1 + T_2f$	0.117	0.412	0.918	1.638	2.321	3.752	5.147	6.301	8.880		
$T_2 - T_2f$	—	—	0.025	0.049	0.073	0.150	0.175	0.236	0.370		

that this percentage is a lower limit based only on absolute measurements of identified compounds.

A lower limit for the amount of carbon incorporated via C_1 plus C_3 carboxylation is obtained by plotting T_2—T_2f (Fig. 7). The minimum rate of this incorporation is about 0.4 μmoles/min/ml algae, or about 3 % of the total. Note that this value is

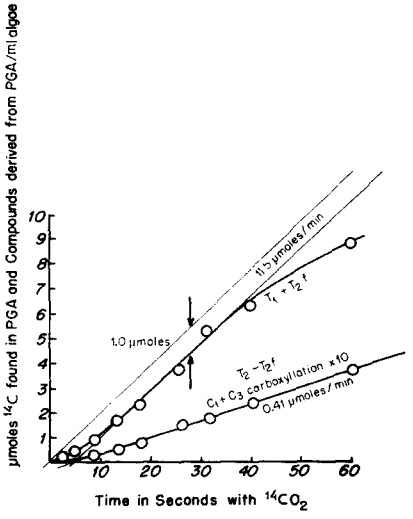


Fig. 7. Appearance of ^{14}C in compounds derived from PGA and in compounds derived from $C_1 + C_3$ carboxylation in *Chlorella pyrenoidosa vs.* time of photosynthesis with $^{14}CO_2$.

for the actual introduction of CO_2 and does not include the carbon derived from PGA (T_2f). The rate of incorporation of ^{14}C into these three compounds thus accounts for about 4 times 3, or 12 % of the total in this experiment. Other experiments indicate that the relative contribution of C_3–C_1 carboxylation varies considerably and tends to be higher (up to 3 times that reported in this case) when the rate of CO_2 fixation is greater and when amino acid synthesis is more rapid. In addition to the three compounds listed here, other substances may be derived in part from C_1–C_3 carboxylation, such as glutamic acid and citric acid, discussed below.

While at least 73 % of the total rate of fixation of carbon has thus been shown to be due to the carbon reduction cycle and C_1–C_3 addition, there is no indication of any other significant fixation pathway. In Fig. 8 the ^{14}C found in glutamic acid and in citric acid is shown. Could this labeling of glutamic acid be the result of a carboxylation of γ-aminobutyric acid? The maximum rate of labeling of glutamic acid and in citric acid is shown. The maximum rate of labeling of glutamic acid is about 0.7 μmoles/min or 4.5 % of all ^{14}C fixed. Since this rate is found between 5 and 20 min, it probably represents labeling of all five carbon atoms of glutamic acid, because the precursors are surely at least partially labeled after 5 min. The labeling due to carboxylation reaction would be expected to begin during the first 30 sec, if one is to judge by the other known carboxylation reactions which were discussed earlier. Yet, after the first 31.5 sec, the glutamic acid contains only 0.02 μmoles of ^{14}C. Between 40 and 60 sec, its labeling rate is only 0.2 μmoles/min. Moreover, γ-aminobutyric acid itself would have to be synthesized from CO_2 (by some as yet

unknown route), if it were a precursor to glutamic acid, and would have to be appreciably labeled by the time glutamic acid reaches its maximum labeling rate. Yet we can detect no radiocarbon in γ-aminobutyric acid in this experiment or in others of this series, even after the algae have been exposed to $^{14}CO_2$ for 10 min. Clearly, little if any of the labeled glutamic acid formed in our experiments is made by

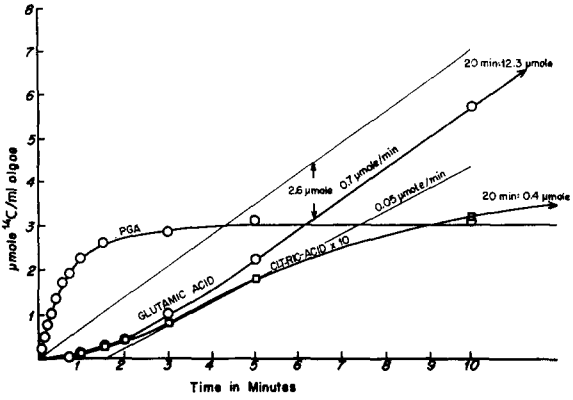


Fig. 8. Appearance of ^{14}C in PGA, glutamic acid and citric acid in *Chlorella pyrenoidosa vs.* time of photosynthesis with $^{14}CO_2$.

carboxylation of γ-aminobutyric acid. Rather, it must arise from other intermediate substances such as those formed by the two carboxylation mechanisms already discussed.

Note, however, that the rate of labeling of citric acid is by far too small to permit it to be the precursor of the labeled glutamic acid in any sequence such as:

$$\text{oxaloacetic acid} + \text{acetyl coenzyme A} \longrightarrow \text{citric acid} \rightarrow\rightarrow\rightarrow$$
$$\alpha\text{-oxoglutaric acid} + CO_2 \longrightarrow \text{glutamic acid}$$

Steady state Expt. 28

All the results described thus far were obtained in an experiment (steady state 18) in which the nutrient solution, though not automatically replenished, was sufficient to maintain the rate of photosynthesis at a nearly constant level during the course of the experiment. The results of steady state Expt. 28, in which the nutrient solution was replenished during the course of the experiment led to the same conclusions.

TABLE VI

COMPARISON OF STEADY STATE EXPERIMENTS 18 AND 28

Experiment	*CO_2 uptake μmoles/min/ml algae*	*Rate of appearance of ^{14}C in compounds on chromatograms (20–40 sec)*	*RuDP saturation at 40 sec*	*PGA residual* carbon saturation according to*	
				Reaction D	*Reaction L*
18	15.5	13	0.53	0.57	0.46
28	19.5	17–18	0.38	0.43	0.28

* See subsequent discussion for explanation of the term "residual". The degree of saturation at 40 sec is obtained by dividing the measured value of ^{14}C in the compound at 40 sec by the saturation level of ^{14}C in the compound (or residual atoms) after 10 min exposure of the algae to $^{14}CO_2$.

These results are summarized and compared with steady state Expt. 18 in Table VI. Though not shown in the table, the maximum rate of appearance of ^{14}C in observable compounds derived from the carboxylation reaction leading to PGA (the carbon reduction cycle) was 70 to 90 % of the externally measured rate of ^{14}C uptake.

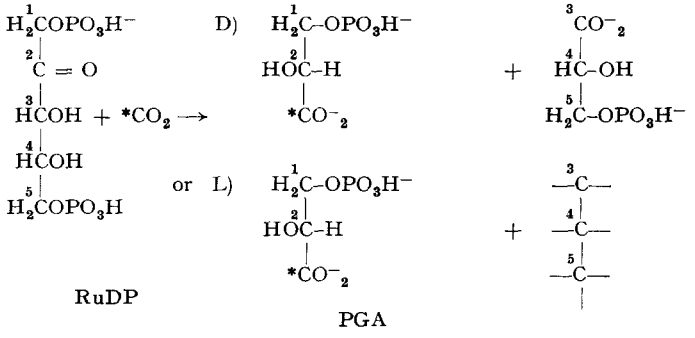
DISCUSSION

When CALVIN AND MASSINI[13] reported the formation of PGA in an overall reaction requiring ribulose diphosphate and CO_2 they proposed that the reaction in the light gave one molecule of PGA and one of triose phosphate but in the dark gave two molecules of PGA. WILSON[14] discussed this possibility further after it was realized that the carboxylation did not involve an intermediate splitting of the ribulose to triose and diose. The dark reaction in whole plants[15] and the reaction in isolated enzyme systems[16,17] was found to give rise to two PGA molecules. Also, it is clear from previous kinetic studies[1,18] of carbon fixation during photosynthesis that the ^{14}C entering the carbon reduction cycle *via* the ribulose carboxylation passes through the carboxyl group of PGA initially. This is consistent with the fact, established for the isolated enzyme system by HORECKER[16], that the CO_2 is bonded to the number two carbon atom of ribulose diphosphate. More recently PARK[19] has shown by means of inhibition studies in broken spinach chloroplasts that ^{14}C entering that system must pass through PGA. That is, PGA is a biochemical intermediate compound—not merely a compound formed by thermal breakdown after the plant is killed.

We shall present here an argument, based on kinetic data, which indicates that the carboxylation of RuDP *in vivo* during photosynthesis gives rise to only one molecule of 3-PGA.

If the ^{14}C which has just entered PGA from $^{14}CO_2$ is subtracted from the total ^{14}C in PGA, the ^{14}C in the remaining carbon atoms of the PGA must all be derived from ribulose diphosphate.

Let us consider the two reactions:



The position of the ^{14}C which has just entered the cycle as $^{14}CO_2$ is indicated by the asterisks. In reaction D, there are five remaining carbon atoms of PGA (numbers 1 to 5) which must be derived from RuDP, while in reaction L there are two such "residual" carbon atoms (numbers 1 and 2). The steady state concentration of PGA in steady state Expt. 18 is 3.0 μmoles of carbon/ml algae, hence the carboxyl carbon concentration is 1.0 μmole of carbon. However, if reaction D is correct, only one-half

of this carboxyl carbon, or 0.5 μmole, is derived immediately from CO_2; the other half (carbon atom 3) comes from RuDP. We shall subtract the ^{14}C due to newly incorporated $^{14}CO_2$ from the total ^{14}C found in PGA at each time and for each of these two cases. The specific radioactivity of the remainder may then be compared with the specific radioactivity of the RuDP from which it must be derived.

In order to make this subtraction it is necessary first to calculate the radiocarbon in the carboxyl group of PGA as a function of the time of exposure of the algae to $^{14}CO_2$. This calculation requires in turn a calculation of the saturation curve of the "CO_2 pool", although this could be assumed to be saturated from the beginning without seriously affecting the results.

Consider the steady state system:

$$CO_2 \xrightarrow{R} \text{Pool 1} \xrightarrow{R} \text{Pool 2} \longrightarrow \text{etc.}$$

Let C_1 and C_2 be the steady state concentrations of Pools 1 and 2 and let x and y be the degrees of saturation with ^{14}C of these pools (respectively) as a function of time of exposure of the algae to $^{14}CO_2$. R is the rate of flow of carbon into the system and through the two pools. It is also assumed in this case that the rates of the back reactions are negligible compared to the rates of the forward reactions.

For a small increment of time, the change in degree of saturation is the difference between the rate of flow of ^{14}C into the pool (R) and the rate of flow of carbon out of the pool (Rx), divided by the size of the pool C_1; $dx/dt = (R—Rx)/C_1$. Integration and determination of the integration constant at $t = 0$ gives $x = 1 - \text{expt}\ (—R/C_1)t)$.

During a small increment of time, the change in degree of saturation of the second pool is the difference between the rate of flow of ^{14}C into the second pool (Rx) and the rate of flow out (Ry) divided by the pool size C_2;

$$y = \frac{Rx - Ry}{C_2} = \frac{R}{C_2}[1 - \exp(-Rt/C_1) - y]$$

Integration and determination of constants at $t = 0$ leads to two solutions, one for the case $C_1 \neq C_2$:

$$y = 1 - \left(\frac{C_1}{C_1 - C_2}\right)\exp(-Rt/C_1) + \left(\frac{C_2}{C_1 - C_2}\right)\exp(-Rt/C_2)$$

and another for the case $C_1 = C_2$:

$$y = 1 - (1 - Rt/C)\exp(-Rt/C)$$

In applying these equations to the data from steady state Expt. 18 we have assumed a value of $C_1 = 1.2$ μmoles for the "CO_2 pool" (Fig. 1) and a value of 0.2 μmoles/sec (= 12 μmoles/min) for R. The resulting values for x are shown by curve A, Fig. 9.

If reaction D is correct, the PGA carboxyl pool arising from newly incorporated CO_2 is 0.5 μmoles and its degree of saturation y is given by curve B, Fig. 9. If reaction L is correct, this pool is 1.0 μmole and the saturation curve y is that shown as curve C. Curve B times 0.5 and curve C times 1.0 give, as a function of time, the respective μmoles of ^{14}C in the PGA carboxyl pool derived directly from CO_2.

The degree of saturation of the residual carbon atoms of PGA (those which are derived from RuDP) may now be calculated by subtracting from the experimentally determined [^{14}C]PGA these values of the CO_2-derived carboxyl (0.5 B for reaction D, 1.0 C for reaction L) and dividing by the pool sizes of the residual carbons (2.5 and

2.0 respectively). The resulting saturation curves are shown in Fig. 10. In the same figure, Curve R is the saturation curve for ribulose diphosphate, obtained by dividing the experimentally determined ^{14}C labeling of RuDP by its steady state concentration, which was 0.36 μmoles/ml algae.

If the carboxylation of RuDP were to lead to the formation of two molecules of PGA (reaction D), then all of the carbon atoms of RuDP must give rise to the "residual" carbon atoms of PGA. The degree of saturation of these residual carbon atoms at no time could exceed the degree of saturation of the carbon atoms of RuDP. Since the calculated values for these residual atoms, (PGA-0.5 B)/2.5, do exceed those of RuDP at all times after 12 sec, reaction D does not appear to be correct. The curve for reaction L does not exceed the saturation of RuDP until about 1 min. In this case, the residual carbon atoms of PGA are derived only from carbon atoms 2 and 3 of RuDP, and thus may exceed the saturation of the average of carbon atoms

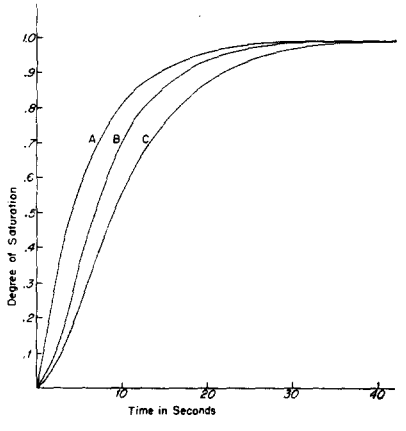


Fig. 9. Degree of saturation (*vs.* time of photosynthesis with $^{14}CO_2$) of "CO_2 pool" and of PGA carboxyl derived immediately from $^{14}CO_2$ according to two proposed carboxylation reactions. Curve A is for "CO_2 pool", curve B is for PGA carboxyl derived immediately from $^{14}CO_2$ according to reaction D, curve C is for PGA carboxyl according to reaction L.

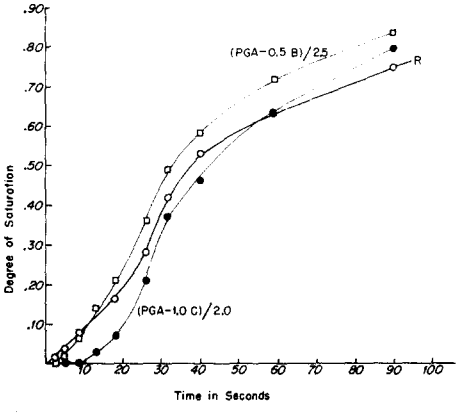


Fig. 10. Degree of saturation of ribulose diphosphate (R) *vs.* time of photosynthesis with $^{14}CO_2$ compared with degrees of saturation of residual carbon atoms of PGA according to two proposed carboxylation reactions.

1, 2, 3, 4, and 5 of RuDP. In fact, this is not surprising, since earlier degradation studies on RuDP[1] showed that, during ^{14}C incorporation in photosynthesis, carbon atom 3 is first labeled, followed by carbon atoms 1 and 2, followed finally by carbon atoms 4 and 5. The saturation curve for the residual PGA carbon atoms according to reaction L is thus about as would be expected.

Note that after 30 sec the carboxyl carbon of PGA would be saturated and the same conclusion could be reached by looking only at the curves from 30 to 90 sec, which are not dependent on the foregoing calculations of CO_2 pool and PGA carboxyl saturation. At these longer times it is sufficient to plot simply the curves for (PGA-0.5)/2.5, (PGA-1.0)/2.0, and RuDP/0.32 all as a function of time.

We conclude, therefore, that the labeling curves for PGA and RuDP in this experiment can best be interpreted as resulting from the occurrence of reaction L. That is, the *in vivo* carboxylation reaction of the carbon reduction cycle during

photosynthesis appears to produce one molecule of PGA and one molecule of some other three carbon compound.

Steady state Expt. 28 gave very similar results, from 10 sec to saturation (see Table VI for comparison at 40 sec).

From these experiments alone we cannot identify this three carbon compound. It could be merely a small pool of PGA itself, tightly bound to an enzyme, or in some other way kept apart from the principal PGA pool. Such a pool of PGA molecules, if sufficiently small (> 0.1 μmole), would not be distinguishable from the other PGA pool by our methods.

Alternatively, the six carbon product of the carboxylation reaction may be reductively split to one molecule of 3-PGA and one molecule of triose phosphate. In either case, the requirement for the reaction leading to PGA and triose phosphate must be light (or cofactors derived from the light reaction), and the intact chloroplast, or some intact sub-unit of the chloroplast, as it occurs naturally in the living cell.

One cannot say at the present time whether or not any of the chloroplasts or chloroplast fragments isolated from broken cells retain the capacity to carry out such a reductive splitting of the six carbon intermediate of the carbon reduction cycle. In such cell-free systems, the carbon reduction cycle may well operate only via the carboxylation reaction leading to two molecules of free 3-PGA. Recently PARK[20] has prepared electron micrographs of chloroplast and chloroplast fragments which had been found by him to have about as high a rate of photosynthetic CO_2 reduction as any such rates reported for cell-free systems. When compared with electron micrographs of chloroplasts in intact cells, these isolated fragments appear to have undergone considerable physical change, particularly in regard to the apparent density of the stroma and spacing between lamellae. It is possible that the reductive carboxylation pathway, if correct, operates only in the unaltered lamellar system by means of some rather direct transfer of photochemically-produced reducing power from the pigmented layer to the carbon reduction cycle.

If two different three carbon compounds are formed *in vivo* in the light by the carboxylation of RuDP, and if these two products are kept separate until they have been converted to triose phosphate, and react with each other to give hexose, then the resulting hexose molecule might be dissimilarly labeled in its two halves, namely carbon atoms 1, 2, and 3, and carbon atoms 4, 5, and 6. Such asymmetry has been reported by GIBBS AND KANDLER[21,22]. However, other explanations of the phenomenon are also consistent with the carbon reduction cycle[3].

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