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**Author**
Platt, Steven G.

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Steven G. Platt, Zvi Plaut, and James A. Bassham

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STEADY-STATE PHOTOSYNTHESIS IN ALFALFA

Steven G. Platt
Lawrence Berkeley Laboratory
Laboratory of Chemical Biodynamics
University of California
Berkeley, California 94720
Analysis of Steady-State Photosynthesis in Alfalfa Leaves
Manuscript received date:

Revised manuscript received date:
Steven G. Platt
Western Regional Research Laboratory
Agricultural Research Service
U. S. Department of Agriculture
Berkeley, California 94710

and

Zvi Plaut and James A. Bassham
Laboratory of Chemical Biodynamics
Lawrence Berkeley Laboratory
University of California
Berkeley, California 94720
1 This work was supported in part by the U. S. Energy Resources and Development Administration and in part by the U. S. Department of Agriculture.

2 Present address: Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720.

3 Present address: Agricultural Research Organization, Bet-Dagan, Israel.

4 Abbreviations: LED: leaf exposure device; UDPG: uridine diphosphoglucose; G6P: glucose-6-P; F6P: fructose-6-P; S7P: sedoheptulose-7-P; PGA: 3-phosphoglyceric acid; Gly: glycine; Ser: serine; PEPA: phosphoenolpyruvate; PS: photosynthesis.
ABSTRACT

A method for carrying out kinetic tracer studies of steady-state photosynthesis in whole leaves has been developed. An apparatus that exposes whole leaves to $^{14}$CO$_2$ under steady-state conditions, while allowing individual leaf samples to be removed as a function of time, has been constructed. Labeling data on the incorporation of $^{14}$C into Medicago sativa L. metabolite pools are reported. A carbon dioxide uptake rate of 79 micromoles $^{14}$CO$_2$ per milligram chlorophyll per hour was observed at a CO$_2$ level slightly below that of air. Several actively turning over pools of early and intermediate metabolites including 3-phosphoglyceric acid, glycerate, citrate, and uridine diphosphoglucone, showed label saturation after approximately 10 to 20 minutes of photosynthesis with $^{14}$CO$_2$ under steady-state conditions. Alanine labeling increased more rapidly at first, and then at a lower rate as saturation was approached. Sucrose was a major product of photosynthesis and label saturation of the sucrose pool was not observed. Labeled carbon appeared rapidly in secondary metabolites. The steady-state apparatus used has numerous advantages including leaf temperature control, protection against leaf dehydration, high illumination, known $^{14}$CO$_2$ specific activity, and provision for control and adjustment of $^{14}$CO$_2$ concentration. Furthermore, the apparatus allows for experiments of long duration, and for sufficient sample points to clearly define the metabolic steady-state.
Knowledge of the mechanism and control of photosynthetic carbon metabolism has increased substantially during the past two decades (1,2,18). Much of that knowledge was obtained from investigations of in vivo steady-state photosynthesis with radioactive carbon dioxide ($^{14}$CO$_2$) by unicellular algae and isolated chloroplasts. Steady-state pool sizes have indicated probable sites of enzymatic regulation (5). Perturbed steady-state experiments have further revealed the nature of metabolic regulation in plants (2). Changes in labeling rates during perturbed steady-state photosynthesis have helped to reveal changes in the overall direction of carbon flow to metabolic products (9).

Steady-state techniques have not as yet been generally applied to the investigation of the complex metabolism of photosynthesizing whole leaves. Leafy plants, such as alfalfa, are a possible source of considerable amounts of protein for direct human consumption (7,13). Ultimately an understanding of regulation in leaves could allow for manipulation of their metabolic processes to cause enhanced production of protein (1), and diminishing of the detrimental effects of the process of photorespiration on plant productivity (17,18).

In this paper we report on the experimental apparatus and procedures we have utilized to obtain and follow steady-state photosynthesis with radioactive carbon dioxide by whole leaves. Kinetic tracer studies of carbon dioxide fixation in higher plant leaves and leaf discs have been previously reported (14,15,16). The method reported here provides for more sample points, known $^{14}$CO$_2$ specific activity, leaf temperature control, longer exposure time, high illumination, and maintenance of a constant level of $^{14}$CO$_2$ during the course of an experiment. The metabolic steady-state can be more clearly defined than in previously reported work.
MATERIALS AND METHODS

Plant Material. Seeds of alfalfa (*Medicago sativa* L., var. El Unico) were planted in five inches of vermiculite in flats. The plants were grown in a chamber at 3000 ft-c with an 8 hr light period at 20 C, and a 16 hr dark period at 15 C. Plants were fertilized with modified Hoagland's solution. Leaflets were selected from 6 week old plants. For experimental purposes leaflets were excised with their petioles immersed under water after two hours of light. Only the central leaflets from the second through fourth unfolded leaves were utilized.

Steady-State System. The gas circulation system is similar to that described previously (3), with several modifications. The modified system is shown in Figure 1. The $^{14}$CO$_2$ analyzer is an ionization chamber monitored by a Cary 401 vibrating reed electrometer. The $^{12}$CO$_2$ analyzer is a Beckman 315B non-dispersive infrared apparatus. The O$_2$ analyzer is an S-3A Applied Electrochemistry device. The outputs from the $^{12}$CO$_2$, $^{14}$CO$_2$, and O$_2$ monitors are continuously recorded. A small Dyna-Vac diaphragm pump is used to recirculate the gas.

$^{14}$CO$_2$ Supply System. The $^{14}$CO$_2$ in the system must be continuously replenished to maintain its concentration and steady-state conditions. The system (shown in Fig. 2) consists of a brass storage cylinder with a large diameter inlet pathway (path A) used for filling the cylinder with labeled CO$_2$. The cylinder is cooled in liquid nitrogen and the $^{14}$CO$_2$ enters by diffusion at reduced pressure. An outlet pathway (path B) incorporating a low flow pressure regulator is provided for release of CO$_2$ from the cylinder into the steady-state apparatus. The system is similar to one designed by Parker (12). In use, the Millafflow micro-metering regulator is adjusted...
to 22 inches of water outlet pressure. The gas supply apparatus is filled with 21% \(^{14}\text{CO}_2\) in nitrogen. This concentration keeps the oxygen level in the steady-state system approximately constant as \(\text{CO}_2\) is introduced to the leaf exposure device at a low flow rate sufficient to maintain the \(^{14}\text{CO}_2\) concentration as shown by the steady-state apparatus recorder. Labeled gas is admitted to the steady-state system through a T in the gas recirculation line. The brass cylinder can contain sufficient \(^{14}\text{CO}_2\) to conduct several whole leaf photosynthesis experiments and therefore eliminates the need for independent production of \(^{14}\text{CO}_2\) for use in each experiment. Furthermore, the supply system allows for any desired concentration of labeled carbon dioxide in the leaf exposure device.

\(^{12}\text{CO}_2\) Supply System. When necessary \(^{12}\text{CO}_2\) is supplied from a cylinder of 21% \(^{12}\text{CO}_2\) in nitrogen. A regulator identical to that in Figure 2 is used.

Steady-State Leaf Exposure Device (LED)\(^4\). The leaf exposure device (see Figures 3,4,5) provides for steady-state exposure of whole leaves to labeled carbon dioxide. The apparatus holds up to sixty alfalfa leaves. The leaves are removable as groups of four leaves at timed intervals. The leaf exposure device (LED) is constructed from transparent Plexiglass. Its outer dimensions are 42 cm x 38 cm x 6.5 cm. The upper and lower chambers of the device are water jackets with the leaf exposure chamber sandwiched between them. Water circulated from a thermostated reservoir enters the jackets through brass inlets (A in Fig. 3). It then flows across the upper and lower surfaces of the leaf chamber and flows out into a 70 cm x 55 cm x 8.3 cm water bath in which the LED is submerged. The upper water jacket is itself covered by a 0.3 cm layer of water. The water returns to the thermostated reservoir through water bath overflow ports. The water circulation system allows control of the temperature
in the leaf exposure chamber. A 0.5 cm greased rubber gasket (E in Fig. 3) is used to form a gas-tight seal between the leaf exposure chamber and the upper water jacket. The entire LED and its associated water bath is enclosed in a plastic box (70 cm x 55 cm x 30 cm) which is vented to a hood.

The leaf exposure chamber has a gas flow entry manifold (four ports enter that manifold, C in Fig. 3, each with an independent flow meter) and a gas flow exit manifold (D in Fig. 3, five ports leave that manifold, four are visible in Fig. 3) on its opposite wall. Gas circulated by the pump shown in Figure 1 flows between the manifolds through the leaf exposure chamber so as to provide for the supply of gas to the upper and lower surfaces of the leaves. Most of the chamber is filled by the carrier disc (36 cm diameter, A in Fig. 4). It is made of two plastic plates separated by several permanently attached small plastic spacers (0.40 cm diameter, 0.15 cm thick). The gap between the plates provides for gas circulation below the leaves. The upper plate has fifteen 6.8 cm diameter compartments, arranged in two concentric circles, in which the leaf holders (B in Fig. 4) fit.

The leaves used are supported in perforated discs (5.3 cm diameter) with space for four leaves each (B in Fig. 4). The leaf holders provide a small well for each leaf so that its petiole can be immersed in water throughout the experiment. Leaf weight measurements show that this procedure and the in-line bubbler (Fig. 1) eliminate leaf weight loss due to dehydration. There are 0.15 cm thick spacers attached to the lower surface of the perforated bed, so as to provide for gas flow below the leaves.

The leaf holders ride on the lower plate of the carrier disc. Their upper surface is flush with the upper surface of the carrier. The rubber gasket between the upper water jacket of the LED, and its lower section (the leaf exposure chamber, and the lower water jacket) provides a gap above the carrier disc that is in contact with the entry and exit gas flow manifolds. That gap provides for gas flow above the leaves. A 0.6 cm x
0.6 cm circular groove is machined into the lower plate of the carrier disc in each compartment in which a leaf holder is placed.

The corners of the leaf exposure chamber contain two plates of plastic, separated by 0.15 cm spacers (E in Fig. 3). The gap made by the spacers provides a pathway from each gas flow manifold to the similar gap between the two plates of the carrier disc.

Two fixed leaf sample removal ports are located on, and with walls extending through, the upper water jacket. Each opening is 6.7 cm in diameter. One opening is centered over each concentric ring of leaf holders.

The schematic diagram shown in Figure 5 should be useful in understanding the structure and operation of the device.

A sliding plastic cylinder (6.3 cm outer diameter, 5.6 cm inner diameter) with 3 O-rings affixed to its outer wall is located in each port in the water jacket. At least one of the two upper O-rings always contacts the wall of the opening in the upper water jacket providing a gas-tight seal. The sliding cylinder is sealed by a plastic plug with an O-ring on its outer surface. The O-ring provides for a gas-tight seal between the plug and the inner surface of the sliding cylinder. The plug has a 0.2 cm diameter hole drilled through it which is sealed by a small rubber stopper. When the sliding cylinder is pushed down it enters the leaf exposure chamber and ultimately contacts the groove in the lower plate of the carrier disc. Its third O-ring provides for a gas-tight seal between that groove and the sliding cylinder. In that position the sliding cylinder isolates one of the leaf holders from the gas flow in the LED and that holder with its sample of four leaves can be removed.

A 0.7 cm diameter steel shaft extends through a rubber seal in the upper water jacket. Its lower end is squared off and fits into a square opening in the center of the carrier disc. A handle on the top of the shaft allows it to be used to rotate the carrier disc so as to bring all the holders under
the ports in the upper water jacket.

When a sample is removed the full procedure is as follows: The sliding cylinder is pushed down around a leaf holder until it seats in the groove in the lower plate of the carrier disc. When this occurs the plastic plug contacts the upper surface of the leaf holder. That leaf holder is now isolated from the atmosphere in the exposure chamber. The rubber stopper in the plastic plug is removed so that the plastic plug can be withdrawn, and the leaf holder is lifted out by means of a forcep (C in Fig. 4). A solid plastic disc (D in Fig. 4) identical in external dimensions to the leaf support is put in its place to prevent an equal volume of room air from entering the LED. The plastic plug is replaced, its hole sealed with the rubber stopper and the sliding cylinder withdrawn. The carrier disc can now be rotated when necessary to remove the next sample.

The overall gas volume of the leaf exposure chamber, associated manifolds, and tubing connecting the LED to the gas circulation system is 2060 ml.

**Light Banks.** Lighting is provided by two banks of 20 w fluorescent lamps. The upper bank is 73 cm x 73 cm (24 lamps), and the lower bank is 60 cm x 60 cm (16 lamps). Light intensity at the upper and lower surfaces of the leaves is 1800 ft-c. This is approximately equivalent to a total illumination intensity of the order of 3600 ft-c from a single light bank above the leaves (10). Verd-A-Ray Criticolor lamps were used (Verd-A-Ray Corp., Toledo, Ohio), operated by a DC fluorescent lamp power supply. The lamps are cooled by blowers circulating room air.

**Photosynthetic CO₂ Fixation.** Sixty leaves were exposed to unlabeled and then labeled carbon dioxide in air by manipulation of the two gas reservoirs (Fig. 1). Leaves were first exposed, in the light, to 0.033% unlabeled CO₂,
which was allowed to decline to 0.028% $^{12}$CO$_2$ after fifteen minutes of photosynthesis. Then, without altering any other environmental variable, the leaves were exposed to 0.027% ± 0.003% $^{14}$CO$_2$ (specific activity 16.6 μCi/μmole). Gas flow was 4 liters per minute. The LED water bath and water jacket temperature was 15°C ± 1°C. The oxygen concentration throughout the experiment was 20% ± 1%. A bubbler filled with deionized water (20°C) was kept in the gas circulation line throughout the experiment. During the $^{12}$CO$_2$ portion of the experiment, 21% $^{12}$CO$_2$ in nitrogen was supplied as necessary to maintain the CO$_2$ level in the system. During the $^{14}$CO$_2$ portion of the experiment, labeled CO$_2$ was added as necessary from the gas supply device shown in Figure 2. Fifteen leaf samples (each containing four leaves) were removed as a function of time following the initiation of photosynthesis with labeled carbon dioxide.

**Treatment of Leaf Samples.** Leaves were frozen by, and stored in, liquid nitrogen immediately upon removal from the experimental apparatus. Each sample was ground in liquid nitrogen in a tissue grinder, and then with 80% ethanol (v/v) in a dry ice-acetone bath. Leaves were then extracted in the dark in 80% ethanol at 3°C, for approximately 2.5 hr. Solutions were centrifuged (IEC centrifuge, 3 min, 2500 rpm). The supernatant was decanted and aliquots were removed, diluted with 80% ethanol, and their chlorophyll content determined by visible spectroscopy (6). The pellet was then further extracted with 20% ethanol (v/v) for 30 min (room temperature). These suspensions were then centrifuged at 2500 rpm for 5 min and the supernatant decanted. Pellets were washed with 0.3N HCl in 80% ethanol, dried over silica gel in a vacuum desiccator, and combusted (Packard Automatic Combustion Apparatus) to give data on $^{14}$CO$_2$ fixation into insoluble material. Aliquots of the 20% and 80% ethanol extracts were acidified with formic acid, dried and counted by liquid scintillation to determine fixation into soluble material. Data from both
extracts were combined to give total fixation into soluble materials. Aliquots of the 20% and 80% ethanol extracts were analyzed by paper chromatography (9). Chromatography was conducted for both 24 and 48 hr in each direction. Spots were located by radioautography. Identification of major labeled metabolites was carried out by co-chromatography with unlabeled carrier. The extracts were analyzed for glycolic acid by the paper chromatographic method of Bassham and Kirk (4). Radioactivity in each compound was determined by automatic Geiger counter (9). Results for the two extracts of each sample were combined. The $^{14}$CO$_2$ fixation results were expressed on the basis of microgram atoms $^{14}$C fixed (specific activity 16.6 µCi/µg atom) per mg chlorophyll into each metabolite, and into total, soluble, and insoluble products.

RESULTS AND DISCUSSION

The products obtained on the two-dimensional paper chromatograms were similar to those previously reported in other higher plant species (8,11,14, 15,16). Major products formed during nine minutes of fixation with $^{14}$CO$_2$ are shown in a typical radioautograph presented in Figure 6.

The rate of photosynthesis measured by the gas phase analysis instruments during a portion of the $^{12}$CO$_2$ part of the experiment was 90 µmoles C0$_2$/hr · mg Chl at 0.033% CO$_2$. The data obtained for total $^{14}$C incorporation into the leaves and incorporation into soluble products, is shown in Figure 7. The linearity of the total $^{14}$C incorporation plot indicates that steady-state photosynthesis was occurring at a rate of 79 µmoles CO$_2$/hr · mg Chl at 0.027% CO$_2$. The somewhat lower rate than was obtained during a portion of the $^{12}$CO$_2$ part of the experiment is understandable given the slightly lower CO$_2$ concentration.

Figure 8 presents labeling data on several typical products identified. Included are data on an amino acid (alanine), a carbohydrate synthesis
intermediate (UDPG), a tricarboxylic acid cycle intermediate (citrate), a Calvin-Bassham cycle intermediate (PGA), and a probable photorespiration intermediate (glycerate). The uptake pattern shown for these compounds, except alanine, is the same as the classic one found for saturation of some actively turning over metabolite pools during steady-state photosynthesis in algae (1,2). When photosynthesis with $^{14}\text{CO}_2$ is initiated, the concentration of labeled material in early metabolic pools rises until they are saturated with $^{14}\text{C}$. At this point the specific activity of those materials is identical to that of the incoming $^{14}\text{CO}_2$. The saturation value level of label in each metabolite is then a direct measure of the amount of material in each metabolite pool.

Saturation of the pools of UDPG, PGA, and glycerate was reached within 10 to 20 min after exposure to CO$_2$; citrate reached saturation somewhat later. Other pools which appeared to reach saturation during the experiment were glycolate, maltose, sugar mono and di phosphates, malate and PEPA. Alanine labeling increased more rapidly at first, and then at a lower rate as saturation was approached.

The label uptake pattern for sucrose is shown in Figure 9. The sucrose pool was increasing in $^{14}\text{C}$ content linearly with time and did not reach apparent label saturation. This result is similar to that found in algae for sucrose by Kanazawa, et al. (9). After the first three minutes of photosynthesis, 7% of the total soluble $^{14}\text{C}$ is found in the alfalfa's sucrose pool. After forty-two minutes of photosynthesis 37% of the total soluble $^{14}\text{C}$ is found in the sucrose pool. This is in accord with non-kinetic data presented by Norris, et al. (11), for other higher plant species. Apparently the end product nature of sucrose production by leaves of plants with non-photosynthetic tissue, prevents the achievement of the type of saturation curves shown in Figure 8.
Examination of the results from the entire set of leaf samples indicates that the steady-state pool sizes of each of the identified Calvin-Bassham cycle metabolites are small relative to the total pool of other soluble metabolites. The $^{14}\text{C}$ appears rapidly in secondary metabolites such as sugars, amino acids, and tricarboxylic acid cycle intermediates. For example, after twelve minutes of photosynthesis with $^{14}\text{CO}_2$, only 2% of the soluble fixed $^{14}\text{C}$ was in the PGA pool. This is in accord with conclusions drawn by Jensen and Bassham from non-steady-state experiments with spinach leaves (8).

Advantages of the steady-state apparatus and techniques we have described include:

1) The capability for taking fifteen samples (of four leaves each) provides enough points to clearly define the metabolic steady-state as well as subsequent transient changes resulting from perturbations of the system.

2) The capability for maintenance of labeled carbon dioxide concentration allows for experiments of long duration. The system has the further capability of allowing for changes to be made in the $^{14}\text{CO}_2$ concentration during an experiment, through use of the labeled gas supply system and steady-state system gas reservoirs.

3) The apparatus provides for leaf temperature control and protection against leaf dehydration during experiments. It also provides for a high level of illumination.

The data obtained indicate that a true steady-state of photosynthetic metabolism was obtained. Also, the use of whole leaves may have helped to overcome some disadvantages encountered with leaf discs, such as the effects of metabolism in injured cells. Future experiments will involve determinations of the effects of specific environmental changes on steady-state photosynthesis in alfalfa leaves.
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LITERATURE CITED


LEGENDS FOR FIGURES

Fig. 1. Steady-state system for studies of photosynthesis in leaves. System components are described in the text.

Fig. 2. Labeled carbon dioxide supply system: (1) brass cylinder, (2) Whitey 316 stainless steel valve SS16DKM4, (3) 400 p.s.i. pressure gauge, (4) Whitey valve SS44F4, (5) Hoke valve 3232M4B, (6) Millaflo micro-metering regulator 41100000. Path A is used to fill system, Path B to release $^{14}$CO$_2$ into the steady-state system.

Fig. 3. Leaf exposure device (LED): (A) brass water inlets, (B) rubber gasket, (C) gas entry manifold, (D) gas exit manifold (E) corner plastic plates.

Fig. 4. Leaf exposure device components: (A) carrier disc, (B) perforated disc leaf holder, (C) forcep used to remove leaf holders, (D) solid plastic disc.

Fig. 5. Cross-sectional view showing the geometry of the leaf exposure device in the region of a single leaf holder centered under a sample removal port: (A) rubber stopper, (B) plastic plug, (C) sliding plastic cylinder, (D) O-rings, (E) upper water jacket, (F) carrier disc, (G) lower water jacket, (H) perforated bed leaf holder with four leaves.

Fig. 6. Radioautograph of two-dimensional chromatogram. Alfalfa photosynthesis with 0.042% $^{14}$CO$_2$ in air (specific activity 54.6 µCi/µmole) for nine minutes; extraction with 80% ethanol (v/v). Samples were chromatographed for 24 hrs in each direction.
Fig. 7. Total photosynthetic $^{14}C$ incorporation, and $^{14}C$ incorporation into 20% and 80% ethanol soluble products, by alfalfa leaflets exposed to 0.027% $^{14}CO_2$ (16.6 μCi/μmole) in air at 3600 ft-c.

Fig. 8. (a) and (b). Incorporation of $^{14}C$ into several metabolites by alfalfa leaflets exposed to 0.027% $^{14}CO_2$ (16.6 μCi/μmole) in air at 3600 ft-c.

Fig. 9. Incorporation of $^{14}C$ into sucrose by alfalfa leaflets exposed to 0.027% $^{14}CO_2$ (16.6 μCi/μmole) in air at 3600 ft-c.
Fig. 1

SUPPLY SYSTEM
MULTI-CHANNEL RECORDER
LEAF EXPOSURE DEVICE

$^{14}\text{CO}_2$
$^{12}\text{CO}_2$

XBL754-5160
Fig. 2

XBL754-5159
Fig. 8
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