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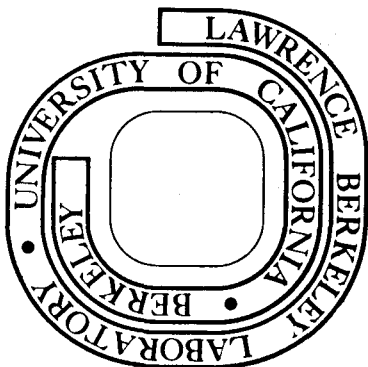
Steven G. Platt

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STEADY-STATE PHOTOSYNTHESIS IN ALFALFA

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Analysis of Steady-State Photosynthesis in Alfalfa Leaves¹

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³More detailed instructions, particularly for construction of the leaf exposure device, are available from the authors at the Laboratory of Chemical Biodynamics, Lawrence Berkeley Laboratory, Berkeley, California 94720, U.S.A.

⁴Abbreviations: LED: leaf exposure device; UDPG: uridine diphosphoglucose; PGA: 3-phosphoglyceric acid; PEPA: phosphoenolpyruvate.

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 $^{14}\text{CO}_2$ 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 $^{14}\text{CO}_2$ 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 diphosphoglucose, showed label saturation after approximately 10 to 20 minutes of photosynthesis with $^{14}\text{CO}_2$ 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 $^{14}\text{CO}_2$ specific radioactivity, and provision for control and adjustment of $^{14}\text{CO}_2$ concentration. The apparatus allows for experiments of long duration, and for sufficient sample points to define clearly the metabolic steady-state.

Knowledge of the mechanism and control of photosynthetic carbon metabolism has increased substantially during the past two decades (1,2,17). Much of that knowledge was obtained from investigations of *in vivo* steady-state photosynthesis with radioactive carbon dioxide ($^{14}\text{CO}_2$) by unicellular algae and isolated chloroplasts. Steady-state pool sizes have indicated probable sites of enzymatic regulation (4). 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 (8).

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 (6,12). 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 (16,17).

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 (13,14,15). The method reported here provides for more sample points, known $^{14}\text{CO}_2$ specific activity, leaf temperature control, longer exposure time, high illumination, and maintenance of a constant level of $^{14}\text{CO}_2$ during the course of an experiment. The metabolic steady-state can be more clearly defined than in previously reported work.

MATERIALS AND METHODS³

Plant Material. Seeds of alfalfa (Medicago sativa L., var. El Unico) were planted in 12 cm 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 closed gas circulation system is similar to that described previously (3), with several modifications. The modified system is shown in Figure 1. The $^{14}\text{CO}_2$ analyzer is an ionization chamber monitored by a Cary 401 vibrating reed electrometer. The $^{12}\text{CO}_2$ analyzer is a Beckman 315B non-dispersive infrared apparatus. The O_2 analyzer is an S-3A Applied Electrochemistry device. The outputs from the $^{12}\text{CO}_2$, $^{14}\text{CO}_2$, and O_2 monitors are continuously recorded. A small Dyna-Vac diaphragm pump is used to recirculate the gas. The reservoirs shown in Figure 1 allow for rapidly changing the composition of the circulating gas.

$^{14}\text{CO}_2$ Supply System. The $^{14}\text{CO}_2$ in the system must be continuously replenished to maintain its concentration and steady-state conditions. The system consists of a brass storage cylinder with a large diameter inlet pathway used for filling the cylinder with labeled CO_2 . The cylinder is cooled in liquid nitrogen and the $^{14}\text{CO}_2$ enters by diffusion at reduced pressure. An outlet pathway incorporating a low flow pressure regulator (Millaflo micro-metering 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 (11). 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 CO_2 is manually 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. The brass cylinder can contain sufficient $^{14}\text{CO}_2$ to conduct several whole leaf photosynthesis experiments and eliminates the need for independent production of $^{14}\text{CO}_2$ for use in each experiment. 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 described above is used.

Steady-State Leaf Exposure Device (LED)⁴. The leaf exposure device (see Fig 2,3 Figures 2,3) 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) was constructed from transparent Plexiglass. The upper and lower chambers of the device are thermostated water jackets with a leaf exposure chamber sandwiched between them. The entire LED and its associated water bath is enclosed in a plastic box which is vented to a hood.

The leaf exposure chamber (overall gas volume 1500 ml) has gas flow entry and exit manifolds. 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 (A in Fig. 2). It is made of two plastic plates separated by several permanently attached small plastic spacers. The gap between the plates provides for gas circulation below the leaves. The upper plate has fifteen compartments, arranged in two concentric circles. The leaves used are supported in perforated discs (leaf holders, B in Fig. 2) with space for four leaves each. The leaf holders fit in the compartments in the carrier disc and 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 for periods of at least 2.5 hr. There are spacers attached to the lower surface of the perforated bed, so as to provide for gas flow below the leaves.

Two fixed leaf sample removal ports (C in Fig. 2; also see Fig. 3) are located on, and with walls extending through, the upper water jacket. One opening is centered over each concentric ring of leaf holders. A steel shaft extends through a rubber seal in the upper water jacket. Its lower end fits into an 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 (see Fig. 3): The sliding cylinder is pushed down around a leaf holder until it seats in a 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. A solid plastic disc identical in external dimensions to the leaf holder 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. Sample removal time is less than fifteen seconds. Samples can be removed at minimum intervals of one to two minutes.

Light Banks. Lighting is provided by two banks of 20 w fluorescent 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 (9). 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 0.038% unlabeled CO₂, which was allowed to decline to 0.028% ¹²CO₂ after fifteen minutes of photosynthesis. This was done so as to permit measurement of the rate of photosynthesis. Then, without altering any other environmental variable, and by manipulation of the gas reservoirs, the leaves were exposed to 0.027% ± 0.003% ¹⁴CO₂ (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 ¹²CO₂ portion of the experiment, 21% ¹²CO₂ in nitrogen was supplied as necessary to maintain the CO₂ level in the system. During the ¹⁴CO₂ portion of the experiment, labeled CO₂

was manually added from the gas supply device as necessary to maintain the gas concentration in the range given above. 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 (5). 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 $^{14}\text{CO}_2$ 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 (8). Two sets of chromatograms were prepared from each extract: in one set, each chromatogram was developed 24 hr in each direction; in the other set, each chromatogram was developed 48 hr in each direction. Radioactive areas were located by radioautography. Identification of major labeled metabolites was carried out by co-chromatography with unlabeled carrier. Radioactivity in each compound was determined by automatic Geiger counter (8). Results

for the two extracts of each sample were combined. The $^{14}\text{CO}_2$ fixation results were expressed on the basis of microgram atoms ^{14}C fixed (specific activity $16.6 \mu\text{Ci}/\mu\text{g atom}$) 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 (7, 14).

The rate of photosynthesis measured by the gas phase analysis instruments during a portion of the $^{12}\text{CO}_2$ part of the experiment was $90 \mu\text{moles CO}_2/\text{hr} \cdot \text{mg Chl}$ at $0.033\% \text{CO}_2$. The data obtained for total ^{14}C incorporation into the leaves, incorporation into soluble products, and incorporation into sucrose is shown in Figure 4. The total ^{14}C incorporation plot indicates that steady-state photosynthesis was occurring at a rate of $79 \mu\text{moles CO}_2/\text{hr} \cdot \text{mg Chl}$ at $0.027\% \text{CO}_2$. The somewhat lower rate than was obtained during a portion of the $^{12}\text{CO}_2$ part of the experiment is expected due to the slightly lower CO_2 concentration.

Figure 5 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 reductive pentose phosphate cycle intermediate (PGA), and a possible photorespiration intermediate (glycerate). The uptake pattern shown for these compounds, except alanine, is the same as that 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}C . From that time on, the specific radioactivity of those materials is identical to that of the incoming $^{14}\text{CO}_2$ (neglecting possible dilution from endogenous carbon sources). The saturation value level of label in each

1 2 0 2 0 4 0 0 0 0

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 $^{14}\text{CO}_2$; citrate reached saturation somewhat later. Other pools which appeared to reach saturation during the experiment were 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 ^{14}C content in the sucrose pool increased linearly with time and did not reach apparent label saturation (Fig. 4). This result is similar to that found with algae by Kanazawa, et al. (8). After the first three minutes of photosynthesis, 7% of the total soluble ^{14}C is found in the alfalfa's sucrose pool. After forty-two minutes of photosynthesis 37% of the total soluble ^{14}C is found in the sucrose pool. This is in accord with non-kinetic data presented by Norris, et al. (10), 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 5.

Examination of the results from the entire set of leaf samples indicates that the steady-state pool sizes of each of the identified reductive pentose phosphate cycle metabolites are small relative to the total pool of other soluble metabolites. The ^{14}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}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 (7).

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 define clearly 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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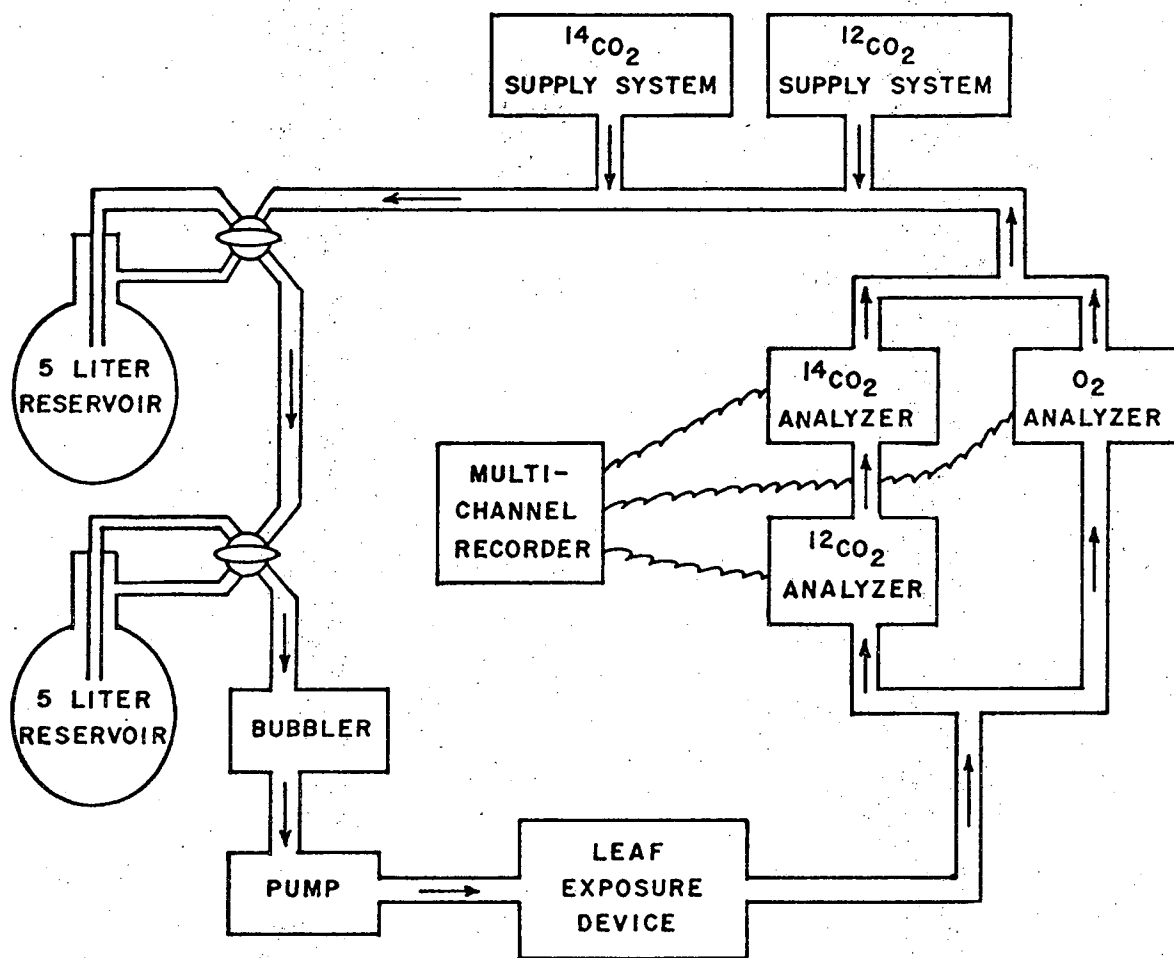
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LEGENDS FOR FIGURES

- Fig. 1. Steady-state system for studies of photosynthesis in leaves. System components are described in the text.
- Fig. 2. Leaf exposure device (LED): (A) carrier disc, (B) perforated disc leaf holder, (C) sample removal port.
- Fig. 3. Cross-sectional view showing the geometry of the leaf exposure device in the region of a 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 disc leaf holder with four leaves.
- Fig. 4. Total photosynthetic ^{14}C incorporation, ^{14}C incorporation into 20% and 80% ethanol soluble products, and ^{14}C incorporation into sucrose, by alfalfa leaflets exposed to 0.027% $^{14}\text{CO}_2$ (16.6 $\mu\text{Ci}/\mu\text{mole}$) in air at 3600 ft-c.
- Fig. 5. (a) and (b). Incorporation of ^{14}C into several metabolites by alfalfa leaflets exposed to 0.027% $^{14}\text{CO}_2$ (16.6 $\mu\text{Ci}/\mu\text{mole}$) in air at 3600 ft-c.



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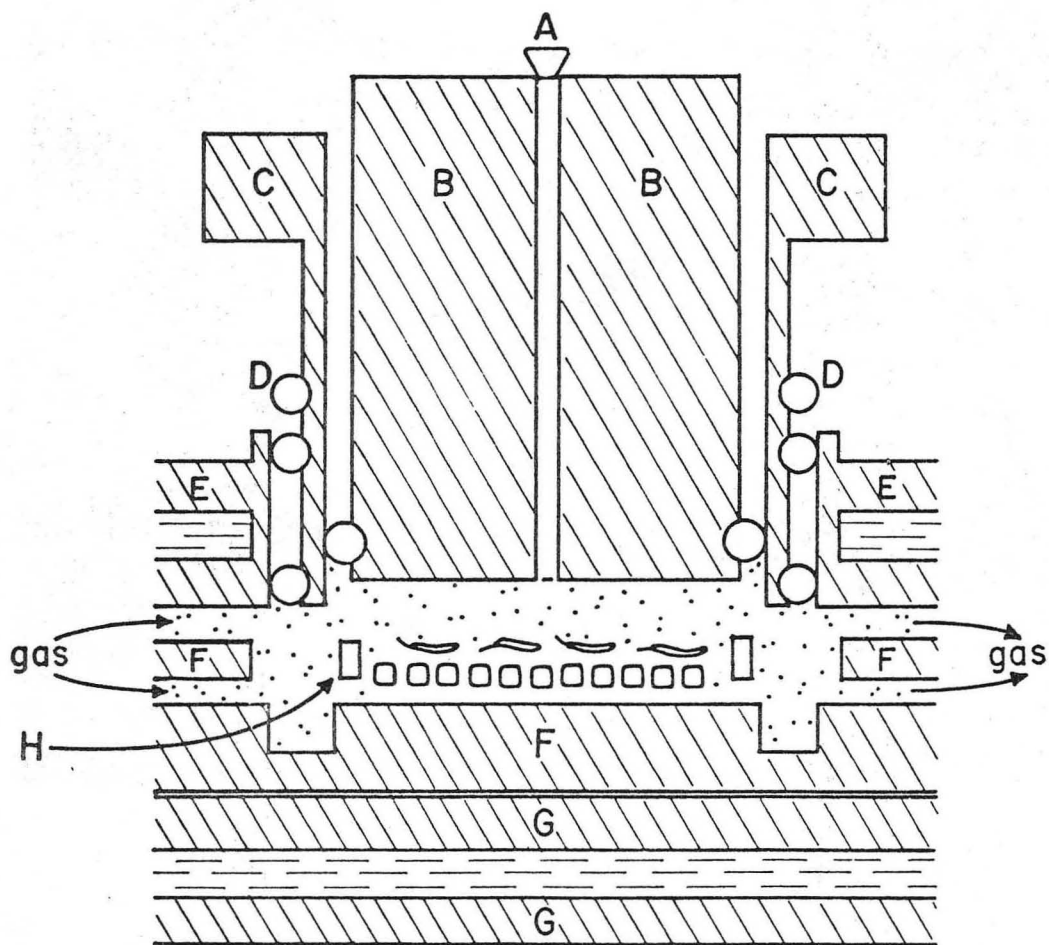
Fig. 1



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Fig. 2

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Fig. 3

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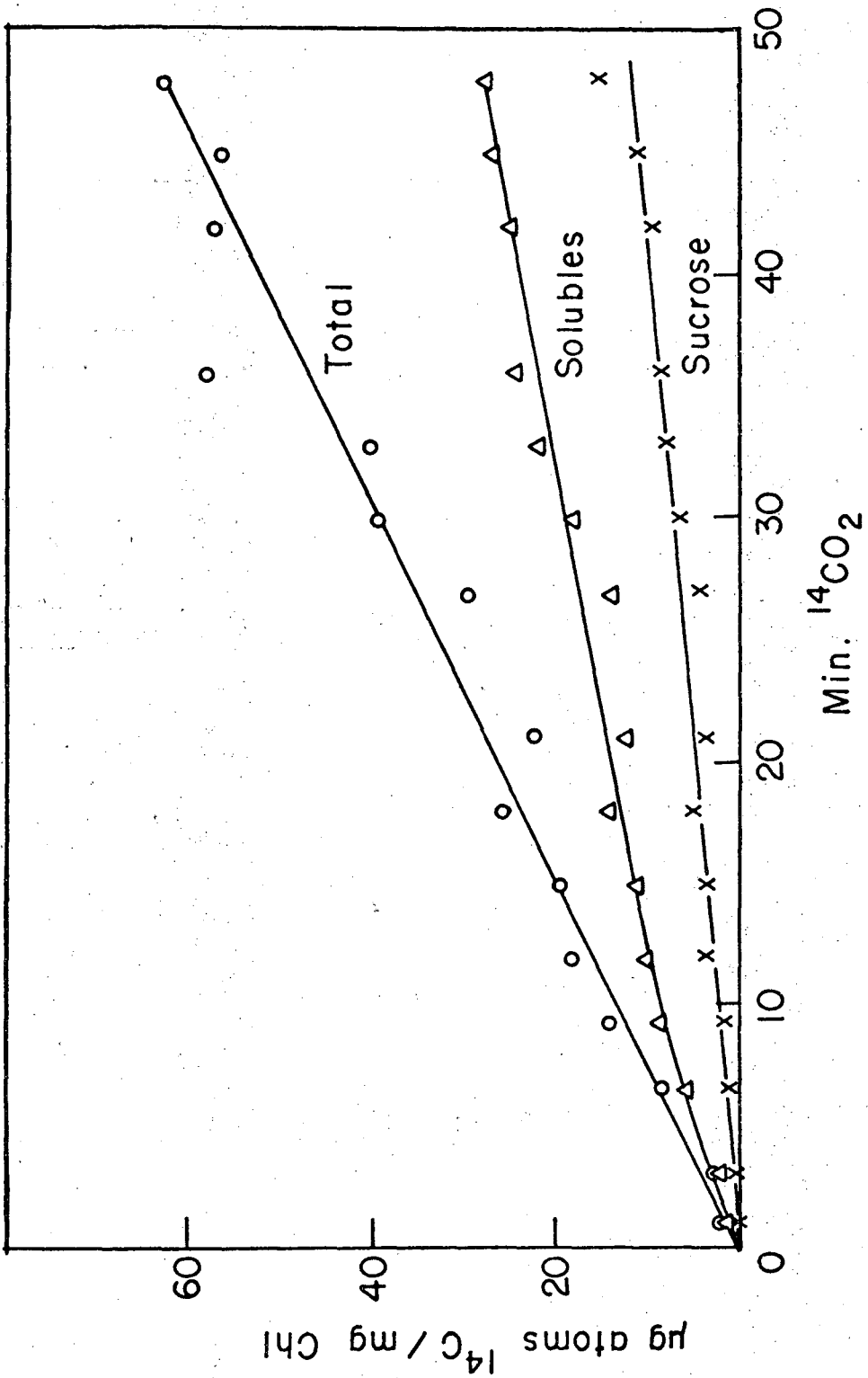
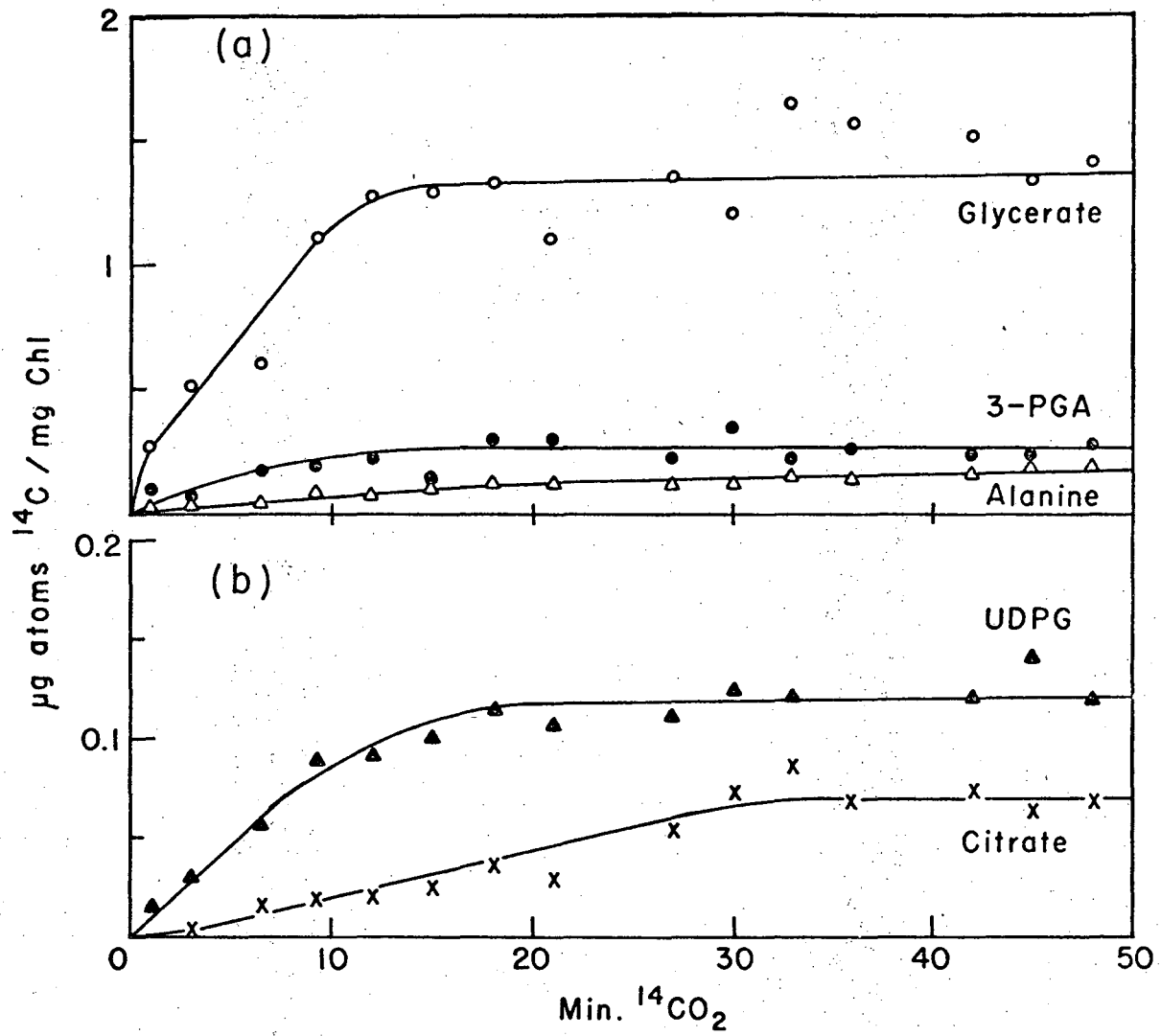


Fig. 4

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Fig. 5

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