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STEADY STATE PHOTOSYNTHESIS IN ALFALFA: EFFECTS OF CARBON DIOXIDE CONCENTRATION

Author
Platt, Steven Gary.

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Steven Gary Platt
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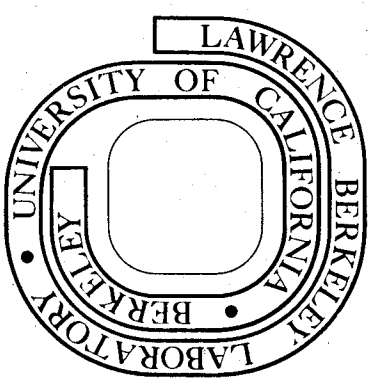

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DISCLAIMER

DEDICATION

To Barbara my wife and to my children Jennifer and Susanna. Without your love and help this thesis could not have been written. It is yours as much as it is mine.

To my parents Irving and Pauline.

STEADY STATE PHOTOSYNTHESIS IN ALFALFA: EFFECTS OF CARBON DIOXIDE CONCENTRATION

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Steven Gary Platt

Laboratory of Chemical Biodynamics
Lawrence Berkeley Laboratory
University of California
Berkeley, California

May 1976

ABSTRACT

Leafy plants such as alfalfa are a possible source of protein for direct human consumption. Ultimately an understanding of carbon metabolism and its regulation in leaves may allow for manipulation of their metabolic processes to cause enhanced production of desirable constituents and increased crop yield. To that end photosynthetic carbon metabolism in whole alfalfa leaflets was investigated using the techniques of steady state and perturbed steady state photosynthesis. Alfalfa gas exchange parameters were also investigated.

The initial step of this study consisted of the development of a method for successfully carrying out kinetic tracer studies of photosynthesis with whole alfalfa leaflets. An apparatus was designed and constructed that exposes leaflets to $^{14}CO_2$ under steady state and perturbed steady state conditions. It provides for removal of individual leaf samples as a function of time. Sufficient sample points are provided to define the metabolic steady state and changes in labeling occurring when environmental conditions are altered. Also developed

were new paper chromatographic separation methods for the isolation of glycine, serine, and glycolate.

Labeling data are reported on the incorporation of ^{14}C into alfalfa metabolite pools during steady state photosynthesis with half saturating $^{14}CO_2$ in air. Many metabolite pools were observed to saturate with ^{14}C. The active pool sizes of the reductive pentose phosphate (RPP) cycle intermediates were small relative to incorporation into total soluble products. The major labeled soluble product was sucrose. Based on the steady state labeling data, conclusions are drawn about carbon flow in alfalfa.

Alfalfa photosynthesis was found to be stimulated by elevated levels of CO_2 and inhibited by O_2. The leaflets had a high CO_2 compensation point, and estimated photorespiration was significant. This is in accord with the idea that alfalfa productivity could be enhanced by atmospheric manipulation.

Given the possible advantages to mankind of a growing alfalfa at elevated CO_2 concentration, the effect of $^{14}CO_2$ concentration on alfalfa photosynthetic metabolism was next investigated using the whole leaflet tracer analysis techniques developed. The $^{14}CO_2$ concentration available to the leaflets was changed from half saturating to saturating and then back to half saturating. Labeling curves were obtained for many metabolites. Increased $^{14}CO_2$ concentration resulted in a doubled rate of photosynthesis. The rate increase was accounted for by increased labeling of both soluble and insoluble products. A large increase in ^{14}C flow through the RPP cycle occurred with only a small absolute increase in the pool sizes of RPP cycle intermediates. Tracer carbon flow to tricarboxylic acid cycle

metabolites and amino acids increased. Sucrose labeling appeared to account for a slightly larger portion of the overall fixation rate at saturating $^{14}CO_2$ vs. half saturating $^{14}CO_2$. The general conclusion is that alfalfa photosynthetic carbon metabolism is well balanced; increased ^{14}C flow occurred at elevated $^{14}CO_2$ to and through most metabolite pools examined. However, glycolate production and ^{14}C flow along the glycolate pathway was inhibited at elevated $^{14}CO_2$. Glycine appeared to be synthesized from glycolate. Glycerate and serine (usually associated with glycolate metabolism) were synthesized predominantly from 3-PGA and not from glycolate. Their production was not inhibited at elevated $^{14}CO_2$. Implications of this finding as regards investigations of photorespiration and glycolate metabolism are discussed.

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ABBREVIATIONS

Acetyl CoA	acetyl coenzyme A
AES	automatic external standardization
Ala	alanine
Asp	aspartate
Chl	chlorophyll
DHAP	dihydroxyacetone phosphate
DPM	disintegrations per minute
EDTA	ethylenediaminetetraacetic acid
F6P	fructose-6-phosphate
ft-c	foot-candle
G6P	glucose-6-phosphate
Glu	glutamate
Gly	glycine
HOAc	acetic acid
αKG	α-ketoglutarate
LED	leaf exposure device
LPM	liters per minute
OAA	oxaloacetate
PEPA	phosphoenolpyruvate
PEP carboxylase	phosphoenolpyruvate carboxylase
PGA, 3-PGA	3-phosphoglycerate
P-glycolate	phosphoglycolate
PhOH	phenol
PS	photosynthesis
Pyr	pyruvate
rpm	revolutions per minute

RPP	reductive pentose phosphate
RuDP	ribulose-1,5-diphosphate
S7P	sedoheptulose-7-phosphate
Ser	serine
TCA	tricarboxylic acid
UDPG	uridine diphosphoglucose
w	watt

CHAPTER I

DEVELOPMENT OF THE METHOD AND ANALYSIS OF STEADY STATE PHOTOSYNTHESIS IN WHOLE ALFALFA LEAFLETS

A. Introduction

Major problems facing the world at this time involve the rising cost of protein and its scarcity. Leafy plants are a possible source of considerable amounts of protein for direct human consumption (Pirie 1966, Edwards et al. 1975). Zelitch (1971) has estimated that photorespiration and dark respiration may together account for the loss of a substantial fraction (50% or more) of the gross photosynthetic assimilation of many crop stands. Placing higher plants with rapid photorespiration in conditions unfavorable for that process led to increases in yield (Zelitch 1971). Ultimately a complete understanding of carbon metabolism and its regulation in leaves may allow for manipulation of their metabolic processes to cause enhanced production of desirable constituents such as protein (Bassham 1971) and diminishing of any detrimental effects of respiration on plant productivity (Zelitch 1971, Wittwer 1974).

Present knowledge of photosynthetic carbon metabolism has developed over a long period of time. By the middle of the nineteenth century, photosynthesis was understood to involve the conversion of carbon dioxide and water, through the utilization of light energy, to oxygen and organic matter containing stored chemical energy (Rabinowitch 1945). In 1931 van Niel's observations of photosynthetic plants and bacteria led him to suggest that the hydrogen used in the photosynthetic reduction of carbon dioxide derived from water (van Niel 1931).

Several years later Hill observed that cell-free chloroplast suspensions could produce oxygen by a photoinduced iron reduction (Hill 1937, Hill and Scarisbrick 1940a,b). This observation gave substantial support to the developing idea that photosynthesis consisted of two separate events:

1) The absorption of light energy, and its use to produce oxygen, high energy reducing agents, and energy storing compounds.

2) The use of the energy storing compounds and reducing agents to carry out carbon dioxide reduction as a dark reaction.

Tracing of the path of dark carbon dioxide reduction required a technique for identifying intermediates of the reduction, and establishing the order of their occurrence. Ruben et al. (1939) attempted to use ^{11}C as a radioactive tracer to follow the path of carbon dioxide reduction, but the progress of that work was limited by the short half-life of ^{11}C and by the lack of relatively rapid, high resolution intermediate separation techniques. By the mid 1940's Ruben and Kamen (1940, 1941) had identified ^{14}C, a long lived isotope of carbon; and the separation technique of paper chromatography had been described (Consden et al. 1944). Through the use of ^{14}C as a tracer, paper chromatography as a metabolite separation technique, and mainly unicellular algae as the plant material, the basic path of photosynthetic carbon reduction by plants was essentially established by Calvin and his coworkers (Bassham 1964, Bassham and Calvin 1957).

As the understanding of the reductive pentose phosphate (RPP) or Calvin cycle developed, interest in the regulation of early carbon metabolism in plants grew. The intermediates of the RPP cycle were seen to be chemically identical to many dark metabolism biosynthetic intermediates. Studies with ^{14}C as tracer indicated that some RPP cycle

intermediates were utilized directly, without prior incorporation into sugars, in the synthesis of such materials as amino acids (Smith et al. 1961). Tracer ^{14}C incorporated during leaf photosynthesis rapidly appeared outside the chloroplasts in compounds such as UDPG, malate, aspartate, alanine, and sucrose (Heber and Willenbrink 1964). Bassham et al. (1964) observed the formation of protein during photosynthesis using newly incorporated ^{14}C. Clearly some sort of regulation of the flow of carbon incorporated by the RPP cycle to different cell metabolites and end products (such as protein, sucrose, amino acids, and organic acids) was necessary. (Transport of newly fixed carbon from chloroplasts to cytoplasm during photosynthesis now appears to consist mainly of the export of DHAP and possibly PGA, which are then converted into the compounds mentioned above outside the chloroplast (Heber 1974)).

Knowledge of the mechanism and control of photosynthetic carbon metabolism has increased substantially during the past two decades (Bassham 1971, 1973, Zelitch 1971). Much of that knowledge was obtained from investigations of in vivo steady state and perturbed steady state photosynthesis with $^{14}CO_2$ by unicellular algae and isolated chloroplasts. In fact, such experiments had proved useful in the initial investigations resulting in the formulation of the RPP cycle (Calvin and Massini 1952). The basic plan of the steady state experiment (Bassham and Calvin 1957, Bassham 1971, Bassham 1973) is to allow plant material to photosynthesize with $^{14}CO_2$ after an initial period of photosynthesis with $^{12}CO_2$. No other experimental variable is changed: The concentration of the carbon dioxide and the specific activity of the $^{14}CO_2$ supplied are held constant. Samples are taken as a function of time and killed. The amount of radioactivity in each metabolic pool is determined. As photosynthesis proceeds the

concentration of labeled material in some active metabolic pools (such as those of the RPP cycle) rises until those pools are saturated with ^{14}C. That is, until the ^{14}C content of a given metabolite is observed to reach a constant value during a steady state photosynthesis experiment. The specific radioactivity of such active metabolic pools is then identical to that of the incoming $^{14}CO_2$ (neglecting possible contributions from endogenous sources). The amount of radioactivity in those metabolites is then a direct measure of the amount of material in each active metabolite pool. Active pool sizes are calculated by dividing the ^{14}C content of the metabolite at saturation by the specific radioactivity of the $^{14}CO_2$ utilized. Active steady state pool sizes under constant environmental conditions can indicate sites of regulation. Such pool sizes can be used in the calculation of the steady state free energy changes of metabolic reactions. Reactions with high negative free energies in photosynthetic cells appear to be regulated reactions (Bassham 1973, Bassham and Krause 1969).

Once some metabolites are seen to be saturated with label under a given environmental condition, the system can be perturbed by causing some environmental change which alters the steady state. Samples are taken, killed, and analyzed as they were prior to the change. The size of a saturated pool responds to changes in the rates of reactions following and preceding it. Changes which are observed in pool sizes can help establish the probable sites of regulation that allow the system to adjust to the new environmental condition (Pedersen et al. 1966a, Bassham 1973).

Some pools may not reach saturation with ^{14}C during a given steady state or perturbed steady state photosynthetic experiment, but may continue to increase in label content as photosynthesis proceeds.

This can occur when a metabolite is an end product of photosynthetic metabolism or whenever the flow of recently fixed carbon through a pool is small in relation to the pool size. Metabolic pools close to the CO_2 fixation step, through which a rapid flow of recently fixed carbon occurs, tend to reach saturation before pools more distant from that step. In steady state labeling experiments, data prior to saturation can be interpreted as follows: Division of the ^{14}C content found in a given metabolite prior to saturation by the specific radioactivity of the entering $^{14}CO_2$ indicates the amount of that metabolite that has arisen from recently fixed $^{14}CO_2$ rather than the active pool size. Values determined in that way permit comparison between the overall rate of $^{14}CO_2$ fixation and its flow into individual compounds. Changes in labeling rates of nonsaturated pools during perturbed steady state photosynthesis help to reveal changes in the overall direction of carbon flow to metabolic products, or changes in the relative rates of carbon flow along various branching pathways, and can thereby reveal sites of metabolic regulation (Kanazawa et al., 1970).

As has been implied above, the calculation of the amount of a metabolite present in a sample of plant material from the amount of ^{14}C found in that metabolite at saturation can underestimate its total concentration. This does not necessarily reduce the value of steady state data. A metabolite may occur in both active and inactive pools, with rapid ^{14}C flow occurring only through the active pool. Division of the ^{14}C content of that metabolite at saturation by the specific radioactivity of the entering $^{14}CO_2$ gives the size of only the active pool of that metabolite, and not its total concentration. The size of an active saturated pool (or the labeling rate of an active pool prior to saturation) can help reveal metabolic pathways and their regulation.

Data on active, saturated pools of amino acids, coexisting with inactive pools of the same metabolites, was used by Smith et al. (1961) and Bassham and Kirk (1964) to help reveal the path of amino acid synthesis during photosynthesis in Chlorella.

An observation that an early photosynthetic metabolite did not reach specific radioactivity equilibrium with the feed $^{14}CO_2$ during a steady state experiment may in itself lead to important deductions concerning plant metabolism. For example, Fock et al. (1974) and Mahon et al. (1974) investigated glycolate pathway metabolism (Tolbert 1973a) using photosynthesizing leaf discs. The specific radioactivity of PGA, glycine and serine did not rise to that of the feed $^{14}CO_2$. The kinetics of labeling of those materials led to the conclusion that endogenous precursors of lower specific radioactivity than PGA were supplying carbon to the glycolate pathway. The possible existence of multiple active pools of PGA was ruled out.

Steady state techniques have until now not been generally applied to the investigation of the mechanism and regulation of the complex metabolism of photosynthesizing whole leaves. That is due in part to the difficulty of exposing whole leaves to $^{14}CO_2$, and sampling them as a function of time. Through investigations of steady state photosynthesis by whole leaves, the interactions of the numerous metabolic pathways of the living leaf could be understood in a more complete way than is possible solely through the study of isolated cell organelles. Examination of photosynthesis by whole leaves should be a valuable aid in integrating the results of studies of cell organelles with knowledge of the metabolism and physiology of higher plants. Such examinations should aid in uncovering unsuspected biochemical interrelationships within the functioning leaf. Studies with whole leaves allow

examination of the effects of physiologically and biochemically active substances, and the effects of environmental perturbations on higher plants in a way that is very representative of the true in vivo situation. It is probable that a direct examination of photosynthesis in whole leaves using $^{14}CO_2$ and steady state techniques will be a productive way of obtaining a sufficient understanding of higher plant metabolism to regulate respiration and protein production in the field.

The specific plant to be used in these investigations is alfalfa. It is a presently grown standard agricultural crop, and established production techniques exist for it. It can produce 3 to 10 times as much protein per acre as corn, wheat, soybean, and rice (Stahmann 1968). Alfalfa can even produce more protein per acre than sorghum-sudan and corn when they are treated as forage crops (Stahmann 1968). Leaf protein has a biological value approaching that of whole milk (Stahmann 1968). A process is already in existence for extracting alfalfa leaf protein in a form potentially suitable for direct human consumption (Edwards et al. 1975). Alfalfa has been chosen for investigation as I believe that it, and other protein rich leafy plants, will have a major impact on the world food economy in the future. Additional knowledge about alfalfa metabolism and its regulation can therefore also have a major impact.

Available evidence indicates that study of steady state photosynthesis by whole excised, instead of attached, leaves will not introduce any serious problems as regards understanding normal leaf metabolism. This is in marked contrast to some techniques in use such as the study of vacuum infiltrated or thinly chopped tissue, practices which can impair or even obliterate leaf photosynthetic gas exchange (MacDonald 1975). Turner and Bidwell (1965) found that the gross rate of

photosynthesis of detached bean leaves was essentially equal to that of attached leaves even after two days of detachment. They also found that numerous external treatments (variations of levels of light, temperature, etc.) applied to other parts of a plant did not affect the gross photosynthetic rate of an attached leaf. It was concluded that the gross rate of photosynthesis of a bean leaf is an attribute of the leaf and not normally affected by the external stimuli applied to the other parts of the plant. Pearce et al. (1969) found that attached and detached alfalfa leaves had similar specific leaf weight (leaf dry weight per unit leaf area) net photosynthesis relationships. Detached leaf material (leaf segments) can accumulate additional photosynthate of more than 50% of its original dry weight before dry weight gain as a function of time begins to deviate from linearity (Setlik and Sestak 1971). The use of whole excised leaves may help to avoid the injury effects observed in leaf discs (Macniol 1976). Finally, little difference was found in the percentage of photosynthetic ^{14}C incorporation into several types of metabolites by detached as opposed to attached tomato leaves (Lee and Whittingham 1974).

In this chapter I report on:

1) The experimental apparatus and techniques I have developed in order to obtain and follow steady state photosynthesis with radioactive CO_2 by excised whole alfalfa leaflets. Kinetic tracer studies of carbon dioxide fixation in higher plants have been previously reported (Vill and Parnik 1974, Tamas and Bidwell 1970, Sawada and Miyachi 1974, Atkins and Canvin 1971). The method developed here provides for more sample points, known $^{14}CO_2$ specific radioactivity, leaf temperature control, leaf dehydration protection, gas recirculation, longer possible exposure time, high illumination, and maintenance or

adjustment of the CO_2 level during the course of an experiment. The metabolic steady state can be more clearly and more meaningfully defined, and the data obtained is more complete, than in previous work. The techniques and equipment I have developed should be useful in obtaining a better understanding of photosynthesis in higher plants, and uncovering the specific effects brought about by environmental or biochemical perturbation.

2) Kinetic data obtained on the incorporation of ^{14}C into various metabolic pools of alfalfa.

B. Materials and Methods

1. 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 growth 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 twice weekly with modified Hoagland's solution. The light source used was fluorescent lamps supplemented by incandescent lamps. For experimental purposes leaflets were excised with their petioles under water after 2-hr of light. Only the central leaflets from the second through fourth unfolded leaves were utilized in the ^{14}C tracer experiment. The fresh weight of the leaflets used was generally 45-65 mg.

2. Steady State Gas Circulation System. The closed gas circulation system used is similar to one described previously by Bassham and Kirk (1964). The system as modified for research with whole leaflets is essentially as shown in Fig. 1. A gas flow bypass with an inline stopcock is provided for the $^{12}CO_2$ analyzer so as to permit adjustment of the gas flow rate through that analyzer. Gas flow through the oxygen analyzer is adjusted by means of an acrylic flowmeter

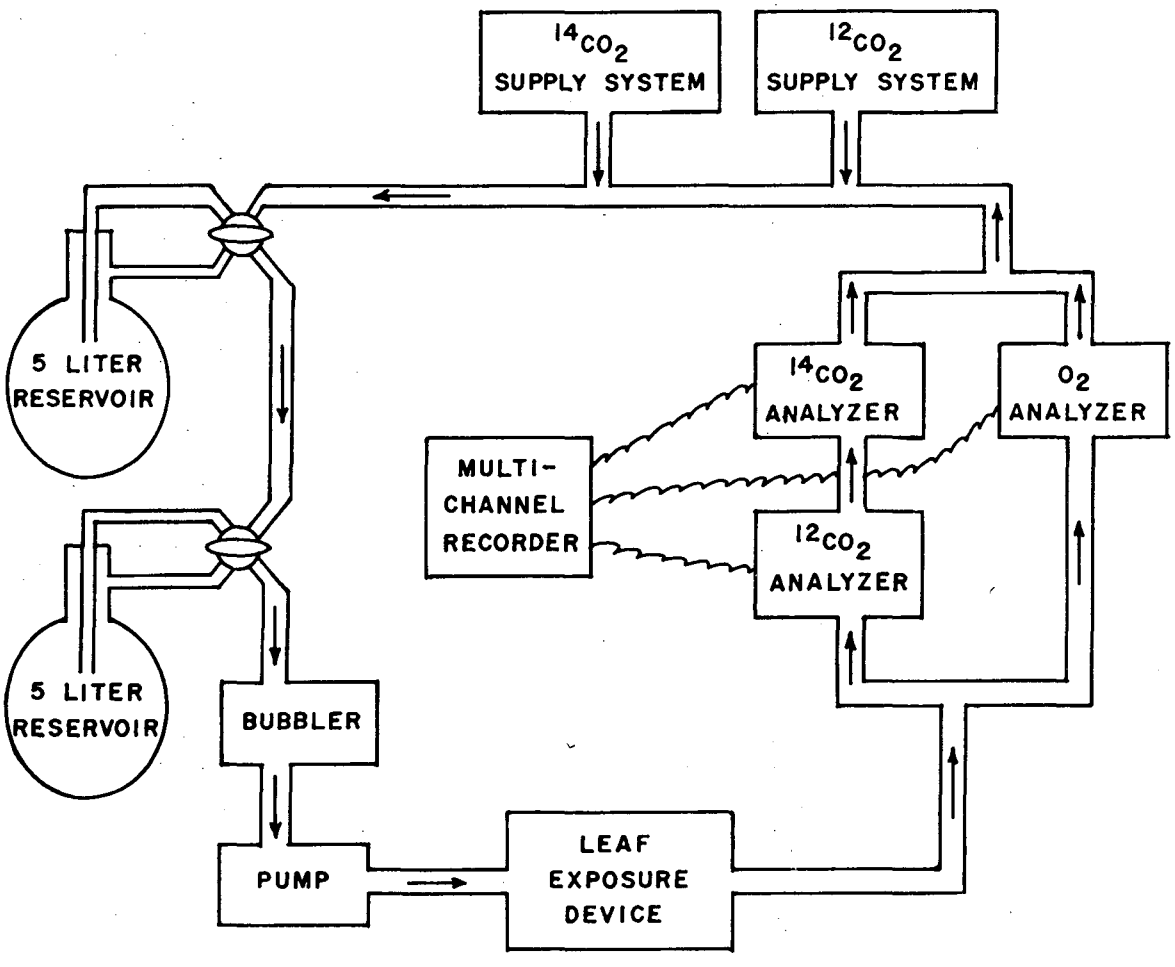

Figure 1. Steady state gas circulation system for studies of photosynthesis in whole leaflets. System components are described in the text.

incorporating a needle valve (Laboratory Supplies Co., Hicksville, N.Y.). 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 Applied Electrochemistry electrochemical cell. The outputs of the $^{12}CO_2$, $^{14}CO_2$, and O_2 monitors are continuously recorded. The $^{12}CO_2$ analyzer is standardized prior to each experiment using gas of known $^{12}CO_2$ content. The O_2 analyzer is standardized against room air, and the calibration zero checked with nitrogen. A small Dyna-Vac diaphragm pump is used to recirculate the gas. A stopcock at the pump provides for variable short circuiting of the pump flow, thereby allowing adjustment of the total gas flow. The reservoirs shown in Fig. 1 (as well as two additional reservoirs of approximately 0.6 and 5 liters) allow for rapid changes in the composition of the circulating gas. In operation the total gas flow in the system was 4 LPM. Gas flow through the O_2 and $^{12}CO_2$ analyzers was 0.2 and 1.0 LPM respectively. Bubblers are attached to two of the large gas reservoirs so that the gas in those reservoirs can be equilibrated with the liquid in the bubblers prior to the start of an experiment. All bubblers and reservoirs can be separately removed from the gas circulation line by operation of four-way stopcocks. The bubblers were designed to provide for a high efficiency of gas-liquid contact with 10 ml of liquid present, a low gas flow back pressure, and to prevent liquid from being carried into the gas circulation tubing at high gas flow rates. A diagram of the type of bubbler used is shown in Fig. 2. The gas volumes of the various system components were determined by observing CO_2 dilution upon addition of a known volume of nitrogen to the system. Those volumes are presented in Table 1.

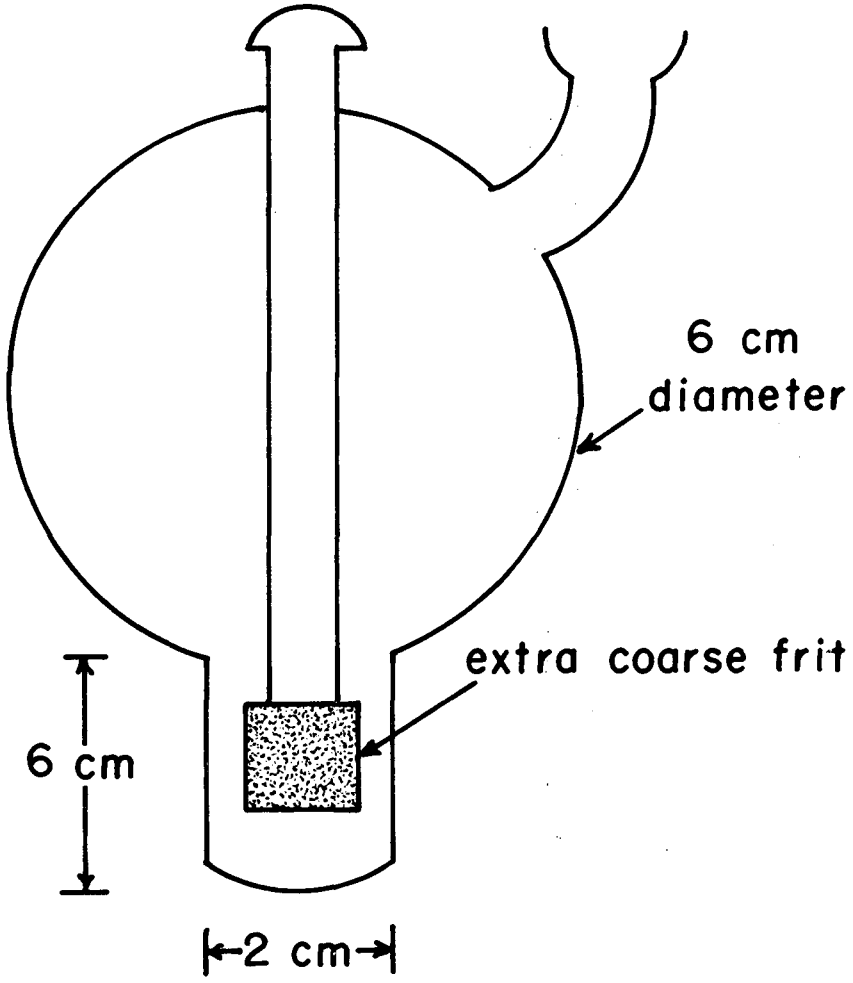

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Figure 2. Steady state gas circulation system bubbler.

Table 1. Gas Volumes of the Components of the Steady State Gas Circulation System

Component	Volume (ml)
1) Gas circulation system tubing (steady state system less all reservoirs, bubblers, and the leaf exposure device)	440
2) Reservoir A (Fig. 1)	5380
3) Reservoir B (Fig. 1)	5580
4) Reservoir D (Fig. 1)	575
5) Reservoir E (Fig. 1)	5210
6) Bubbler (Fig. 2)	120
7) Leaf exposure device	
a) Leaf exposure chamber	1500
b) Total leaf exposure device*	2060

* Includes gas manifolds, tubing to connect manifolds to the gas circulation system, leaf exposure chamber, etc.

3. $^{14}CO_2$ Supply System. The $^{14}CO_2$ in the system must be continuously replenished to maintain its concentration and steady state conditions. For example, given a total system gas volume of 8 liters of 0.033% CO_2, and 60 alfalfa leaflets photosynthesizing at a rate of 100 μmoles CO_2/hr•mg Chl, the leaflets would remove all the CO_2 in the system in under 15 min (extrapolating a constant rate of photosynthesis). The $^{14}CO_2$ supply system developed (Figs. 3a and 3b)

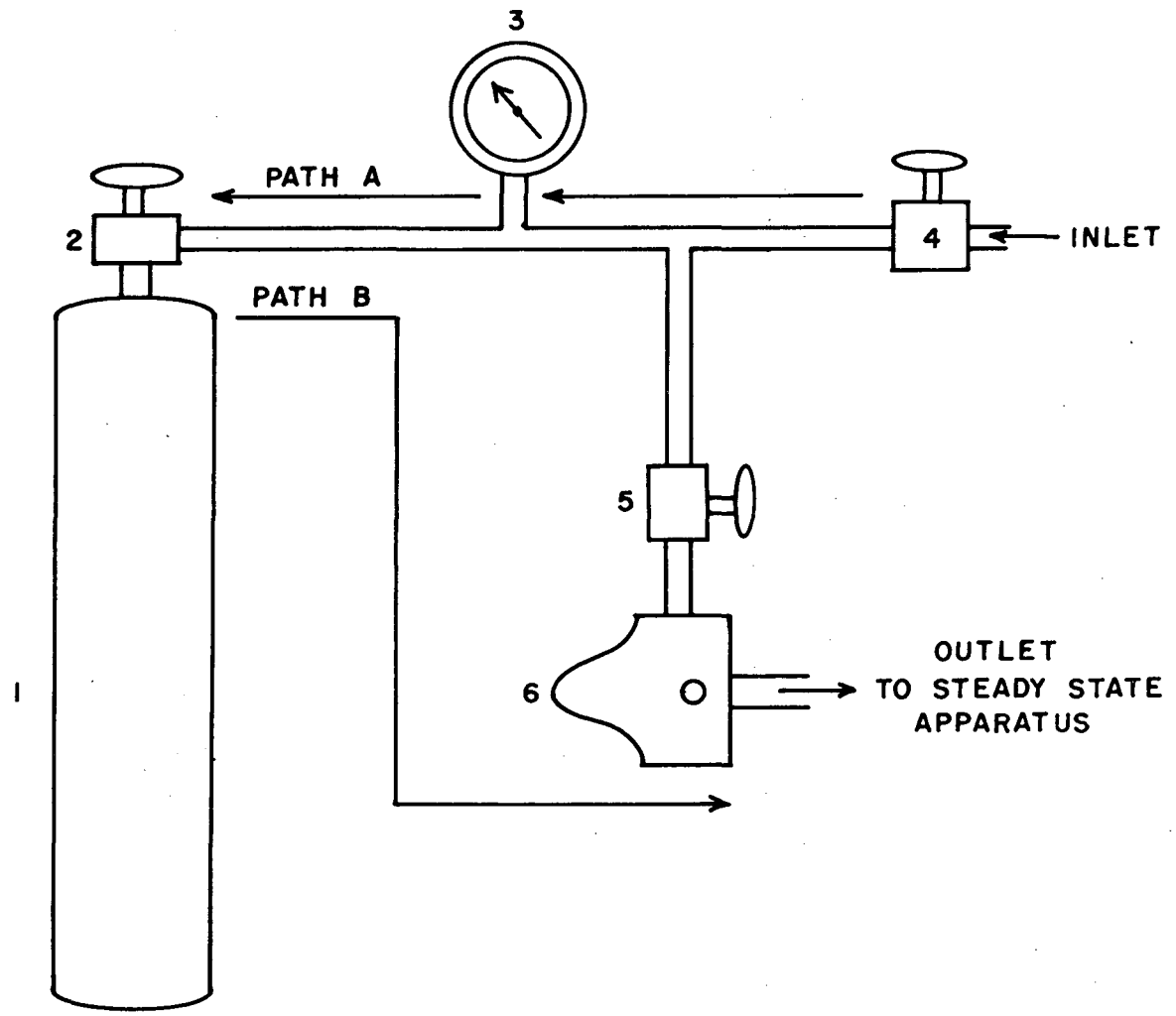

Figure 3a. Labeled carbon dioxide supply system. (1) brass cylinder; (2) Whitey 316 stainless steel valve SS16 DKM4; (3) 400 psi pressure gauge; (4) Whitey valve SS44 F4; (5) Hoke valve 3232M4B; (6) Millaflo micrometering regulator 411000000. Path A is used to fill system, path B to release $^{14}CO_2$ into the steady state gas circulation system.

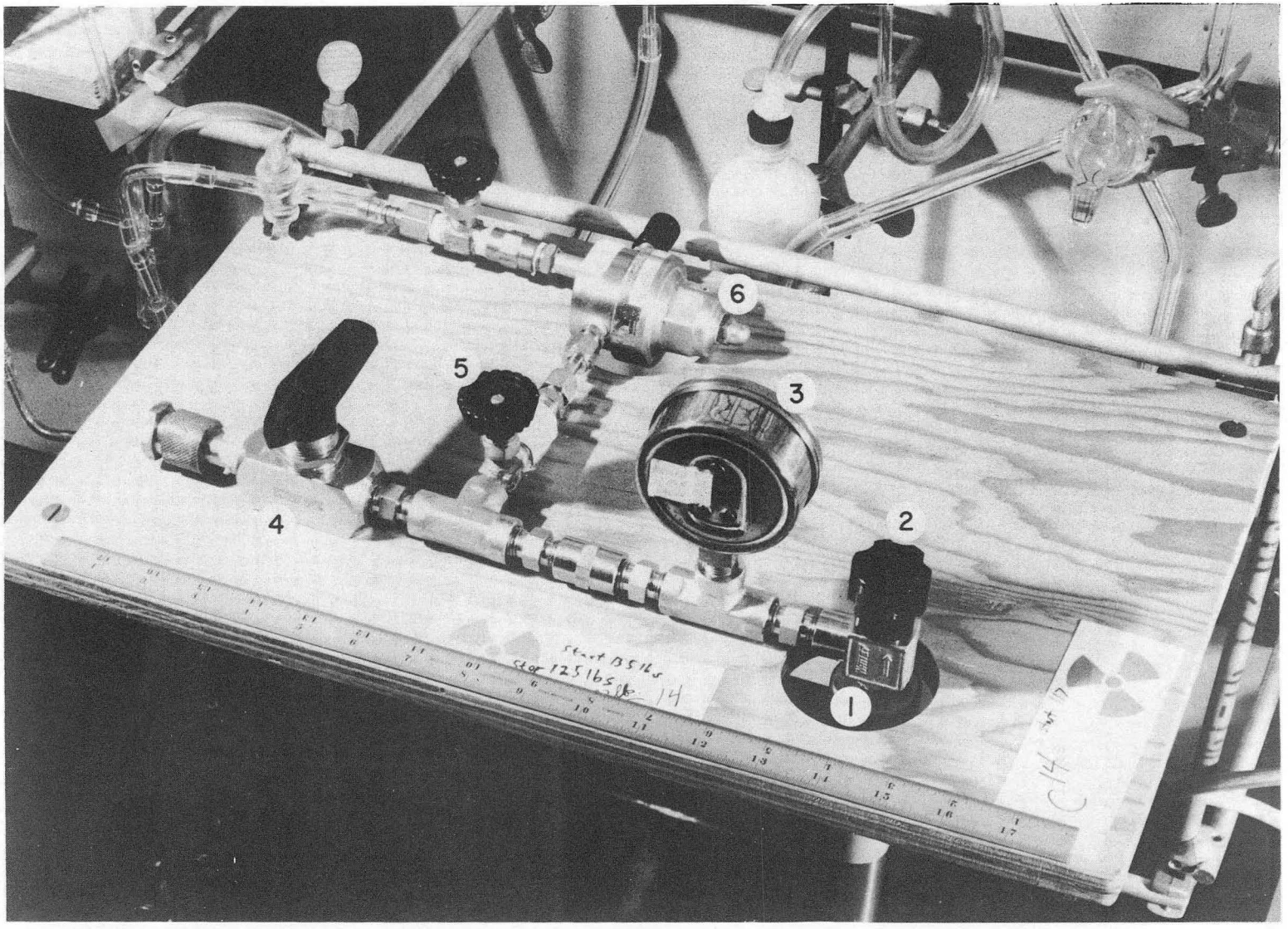

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Figure 3b. Labeled carbon dioxide supply system. Please refer to Fig. 3a.

consists of a brass storage cylinder, with a large diameter inlet pathway (path A, Fig. 3a) for filling the cylinder with labeled CO_2. The cylinder is cooled in liquid nitrogen and the $^{14}CO_2$ (generated from labeled $BaCO_3$) enters by diffusion at reduced pressure (10 μ). An outlet pathway (path B, Fig. 3a) incorporating a low flow pressure regulator (Millaflo micrometering regulator) is provided for release of $^{14}CO_2$ from the cylinder to the steady state apparatus. The gas supply system is similar to one designed by Parker (1971) but with several improvements.

The storage cylinder has an internal volume of approximately 30 ml. The total internal volume of the cylinder and the tubing between valves 2, 4, and 5 (Fig. 3a, b) is 50.8 ml. The supply device has been pressure tested to 2000 psi and can hold a vacuum of less than 10 μ. The Whitey 316 valve provides a relatively unrestricted path for CO_2 diffusion. It also serves to isolate the gas storage cylinder from the pressure gauge when the device is not in use. The pressure gauge is modified for vacuum operation. The Whitey SS 44 F4 provides a large orifice for $^{14}CO_2$ introduction. Valve 5 serves to protect the Millaflo regulator from vacuum when the cylinder is being filled. After introducing $^{14}CO_2$, the cylinder is further charged with $^{12}CO_2$ and nitrogen. The final gas mixture consists of 21% $^{14}CO_2$ (specific radioactivity 16.6 μCi/μmole) in nitrogen. This concentration keeps the oxygen level in the steady state system approximately constant as $^{14}CO_2$ is introduced to that system. Labeled gas is admitted manually to the gas circulation system through a T in the gas recirculation line. It is introduced at a low flow rate sufficient to maintain the $^{14}CO_2$ concentration as shown by the steady state apparatus recorder. In use the Millaflo regulator is adjusted to 22 inches of water outlet pressure. This

allows regulation of a gas flow of less than 1 cc/min. In practice the gas flow is measured by passing it through water and counting the bubbling rate.

The $^{14}CO_2$ gas supply device can contain sufficient $^{14}CO_2$ to conduct several whole leaf photosynthesis experiments, and therefore eliminates the need for independent production of $^{14}CO_2$ for use in each experiment. The supply system allows for any desired concentration of labeled carbon dioxide in the leaf exposure device.

4. $^{12}CO_2$ Supply System. When necessary $^{12}CO_2$ is supplied from a cylinder of 21% $^{12}CO_2$ in nitrogen. A regulator similar to that described above is used.

5. Steady State Leaf Exposure System. The leaf exposure system developed (Fig. 4) permits steady state exposure of whole leaflets to labeled carbon dioxide. It consists of the light banks and the leaf exposure device (LED).

a. Light Banks. Lighting is provided by two banks of 20 w fluorescent lamps (Fig. 4). 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 leaflets (Moss, 1964). 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.

b. Leaf Exposure Device. The plexiglass leaf exposure device (LED, Fig. 5) provides for steady state exposure of up to 60 alfalfa leaflets. The leaflets are removable in groups of four at timed intervals. Its outer dimensions are 42 cm x 38 cm x 6.5 cm. The

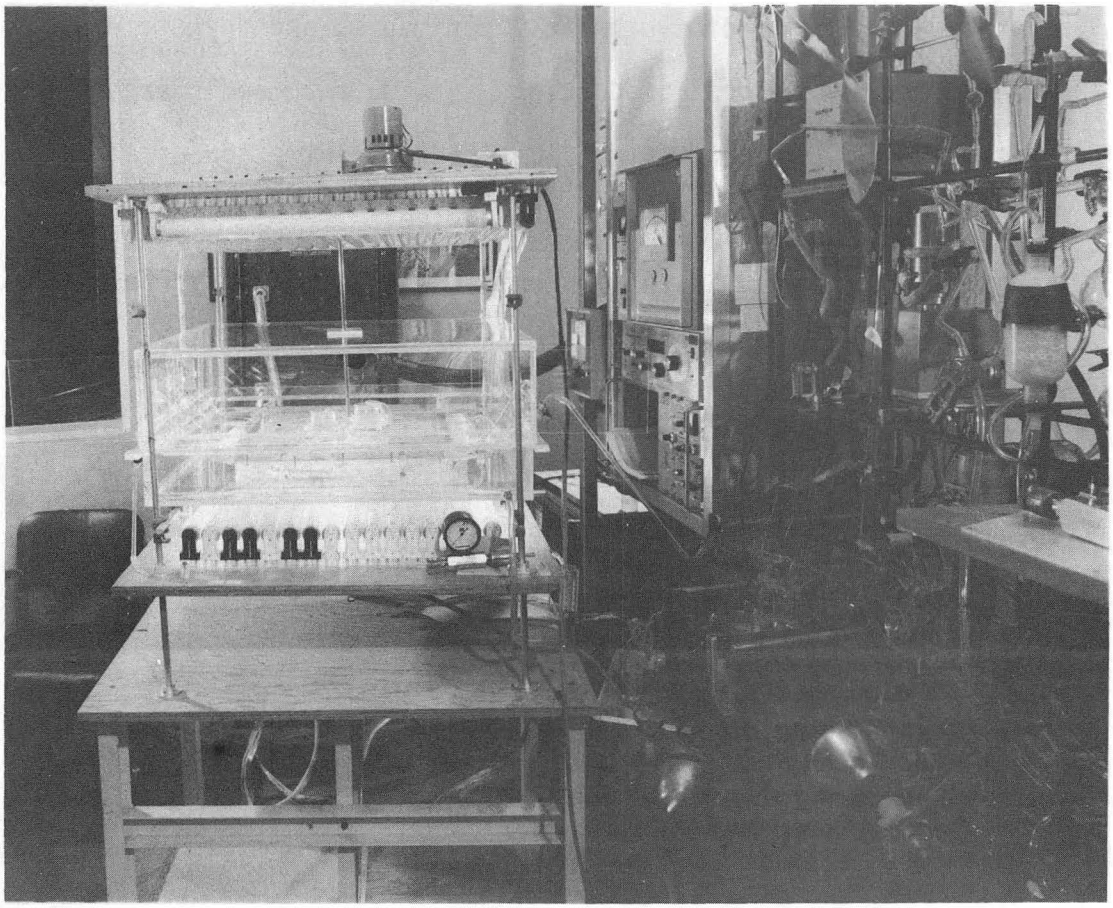

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Figure 4. Steady state leaf exposure system.

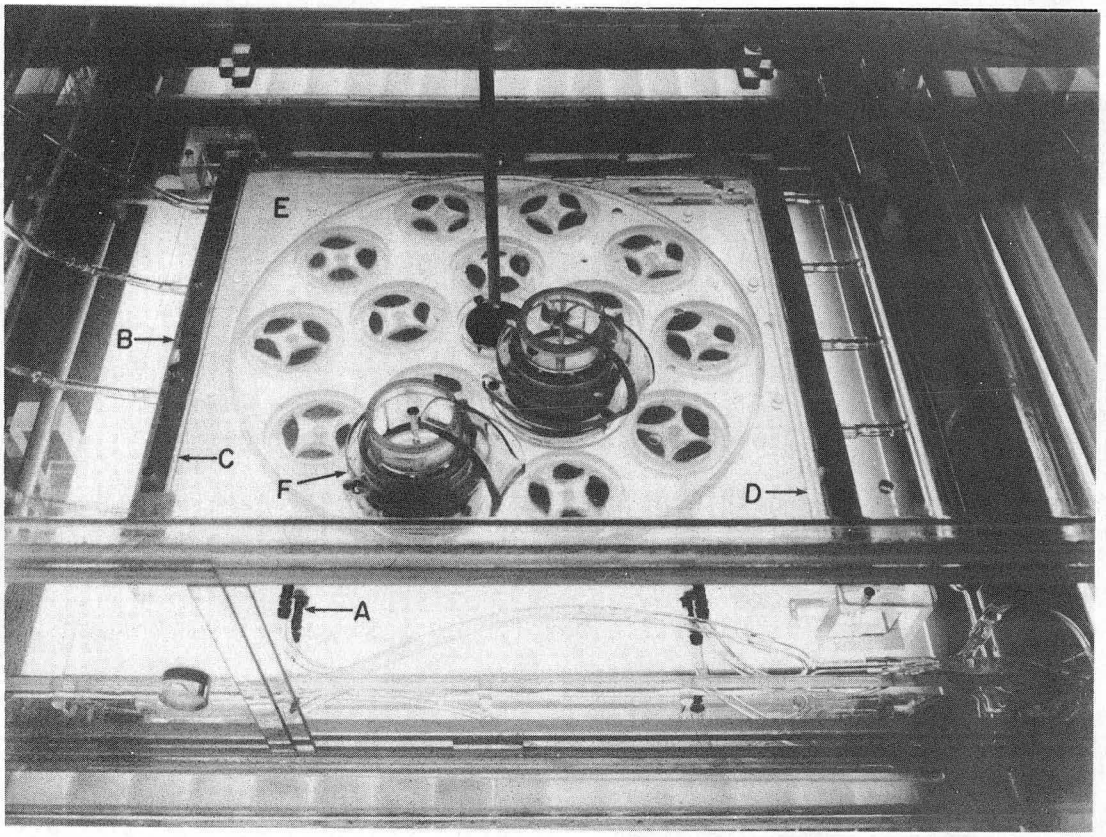

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Figure 5. Leaf exposure device. A: brass water inlets; B: rubber gasket; C: gas entry manifold; D: gas exit manifold; E: corner plastic plates; F: sample removal ports.

upper and lower chambers of the device are water jackets with a leaf exposure chamber sandwiched between them. Water circulated from a thermostated reservoir enters the jackets through brass inlets (Fig. 5A). It then flows across the upper and lower surfaces of the leaf exposure 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. All the water returns to the thermostated reservoir through overflow ports. The water circulation system allows control of the temperature in the leaf exposure chamber. The temperature in the leaf exposure chamber is determined using a thermocouple. A 0.5 cm greased (Apiezon N) rubber gasket (Fig. 5B) 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 has one open side and is vented to a hood.

The leaf exposure chamber (gas volume 1500 ml) has a gas flow entry manifold (four ports enter that manifold, Fig. 5C) and a gas flow exit manifold on its opposite wall (five ports leave that manifold, Fig. 5D). Each gas flow entry port is provided with an independent flowmeter with an adjustable needle valve (Laboratory Supplies Co., Hicksville, N.Y.). Gas circulated by the pump shown in Fig. 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 in diameter, Fig. 6A). It is constructed of two plastic plates separated by several permanently attached plastic spacers (0.4 cm diameter, 0.15 cm thick). The gap between the plates provides for gas circulation below the leaves. The upper plate has fifteen compartments (6.8 cm

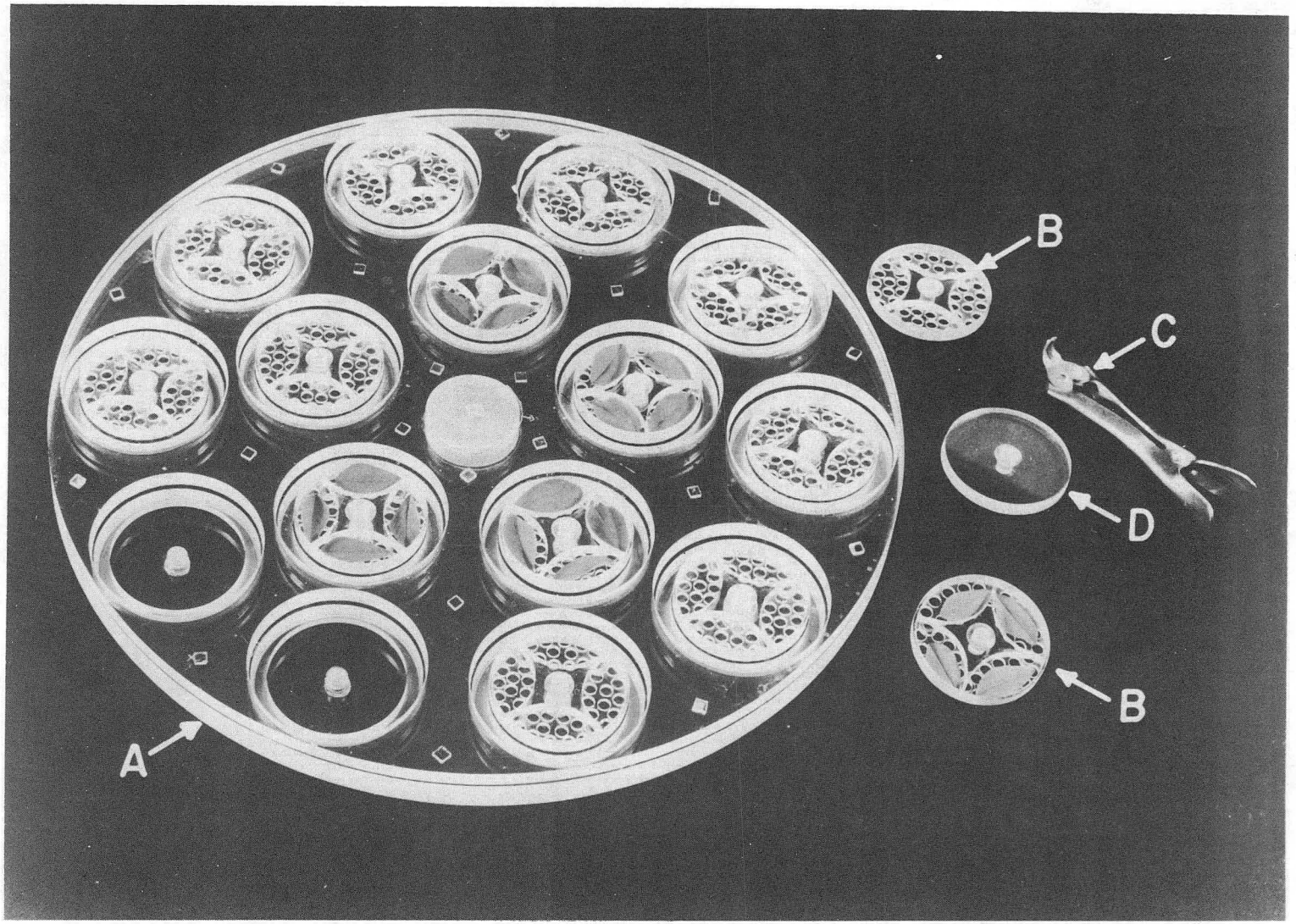

Figure 6. Leaf exposure device components. A: carrier disc; B: perforated disc leaf holder; C: forcep used to remove leaf holder; D: solid plastic disc.

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diameter) arranged in two concentric circles. The leaflets used are supported in perforated discs (leaf holders, Fig. 6B) with space for four leaflets each. The leaf holders fit in the compartments in the carrier disc. They provide a small well for each leaflet so that its petiole can be immersed in water throughout an experiment. Leaf weight measurements show that this procedure in conjunction with the inline bubbler (Fig. 1) eliminates leaf weight loss due to dehydration for periods of at least 2.5 hr. There are 0.15 cm thick spacers attached to the lower surface of the perforated bed so as to provide for gas circulation beneath the leaves.

The leaf holders ride on the lower plate of the carrier disc with their upper surface flush with the upper surface of the carrier disc. 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. This gap provides for gas flow to the upper surfaces of 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 (Fig. 5E) separated by 0.15 cm spacers. 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 sample removal ports are located on, and with walls extending through, the upper water jacket (Fig. 5F). Each opening is 6.7 cm in diameter. One opening is centered over each concentric ring of leaf holders. 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 leaf holders under the ports in the water jacket. The schematic shown in Fig. 7 should be helpful in understanding the construction of the sample removal ports. A sliding plastic cylinder (6.3 cm outer diameter, 5.6 cm inner diameter, Fig. 7C) with 3 O-rings affixed to its outer wall is located in each port in the upper water jacket. At least one of the 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 (Fig. 7B) with an O-ring on its outer surface. That 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 (Fig. 7A). 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 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. 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. That leaf holder with its sample of four leaflets can now be removed without disturbing the atmosphere available to the remaining samples. The rubber stopper in the plastic plug is removed and the plastic plug withdrawn. The leaf holder is lifted out by means of a forcep (Fig. 6C). A solid plastic disc (Fig. 6D) identical in external dimensions to the leaf holder is put in its place to prevent dilution of the chamber atmosphere with

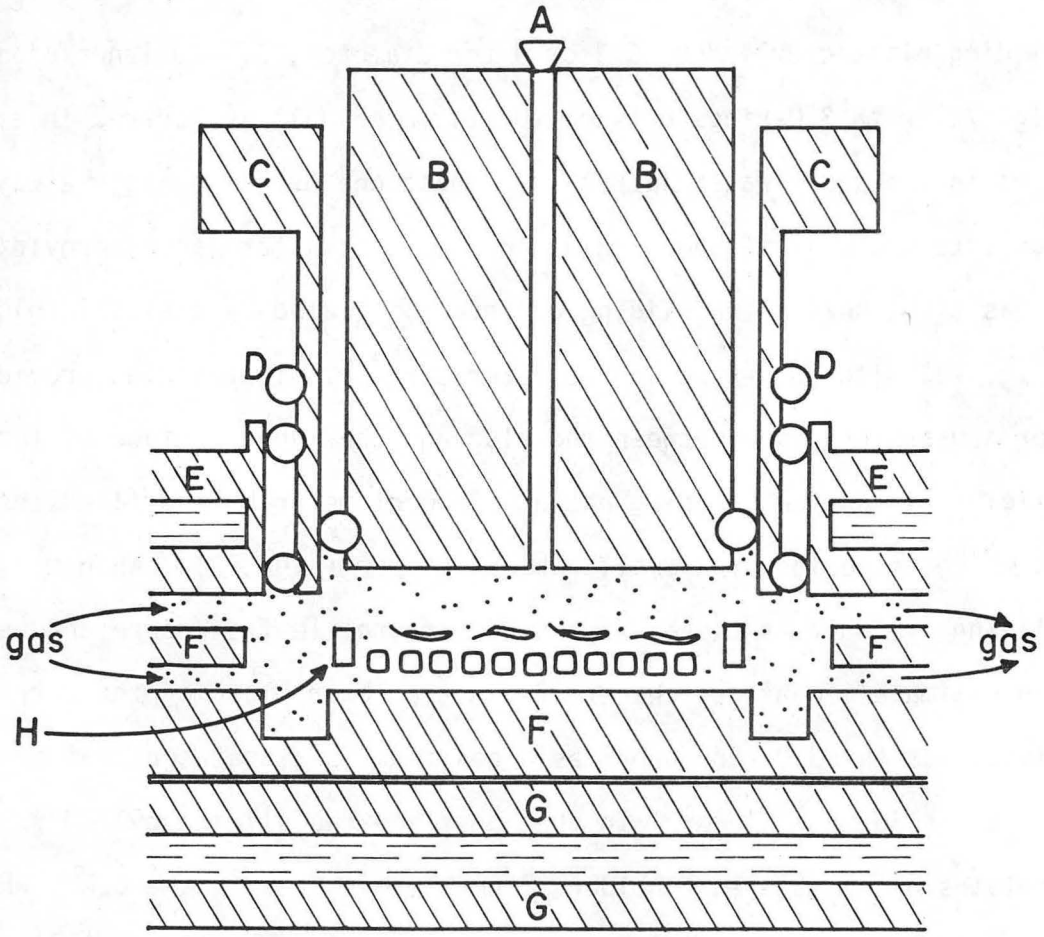

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Figure 7. Cross-sectional view showing the geometry of the leaf exposure device in the region of a leaf holder centered under a sample removal port: A: rubber stopper; B: plastic plug; C: sliding plastic cylinder; D: O-rings; E: upper water jacket; F: carrier disc; G: lower water jacket; H: perforated disc leaf holder with four leaves.

room air. The plastic plug is replaced, its hole sealed with the rubber stopper, and the sliding cylinder is then withdrawn from the leaf exposure chamber. The carrier disc can now be rotated when necessary to remove the next sample. Sample removal time is less than ten seconds. Samples can be removed at minimum intervals of 1 to 2 min.

6. Steady State Photosynthetic $^{14}CO_2$ Fixation. Sixty alfalfa leaflets were exposed to unlabeled and then labeled CO_2 in air by manipulation of the gas reservoirs (Fig. 1). Leaflets were first exposed in the light to 0.038% unlabeled CO_2, which was allowed to decline to 0.028% CO_2 after 15 min of photosynthesis. This permited measurement of the photosynthetic rate. Then without altering any other environmental variable, and by manipulation of the gas reservoirs, the leaflets were exposed to 0.027% $\pm$ 0.003% $^{14}CO_2$ (specific radioactivity 16.6 µCi/µmole). Gas flow was 4 LPM. The LED water bath and water jacket temperature was 15 C $\pm$ 1 C. The leaf exposure chamber temperature was 18 C $\pm$ 1 C. The oxygen concentration throughout the experiment was 20% $\pm$ 1%. A bubbler filled with deionized water (20 C, 10 ml) was kept in the gas circulation line throughout the experiment. During the $^{12}CO_2$ part 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 (specific radioactivity 16.6 µCi/µmole) was manually added from the gas supply device as necessary to maintain the gas concentration in the range given above. Fifteen samples (each containing four leaflets) were removed as a function of time following the initiation of photosynthesis with labeled carbon dioxide.

7. Treatment of Leaflet Samples. Leaflets were frozen in liquid nitrogen immediately upon removal from the experimental apparatus and stored at liquid nitrogen temperature. Each sample was ground in liquid nitrogen in a tissue grinder (Kontes Dupour size 24 tube, size 23 rod), and then with 80% ethyl alcohol (v/v) in a dry ice-acetone bath. Ground leaflets were extracted with magnetic stirring in the dark in 20 ml of 80% ethyl alcohol (v/v) at 3 C for approximately 2.5 hr. Solutions were centrifuged (IEC centrifuge) for 3 min at 2500 rpm. The supernatant was decanted and aliquots were removed, diluted with 80% ethyl alcohol and their Chl content determined by visible spectroscopy using the method of Bruinsma (1963); see additional discussion below. Spectra were corrected for light scattering, and Chl content was taken to be the average of the values obtained from Bruinsma's two equations. The pellet was then further extracted with 5 ml of 20% ethyl alcohol (v/v) for 30 min at room temperature. These suspensions were then centrifuged at 2500 rpm for 5 min and the supernatant decanted. Pellets were washed with 0.3 M HCl in 80% ethyl alcohol, collected by centrifugation at 2500 rpm for 3 min, and dried over silica gel in a vacuum dessicator. They were combusted in a Packard Automatic Combustion Apparatus to determine fixation of $^{14}CO_2$ into insoluble material. CO_2 from combusted samples was collected in Carbo-Sorb and scintillation counted in Permafluor V (Packard Instrument Co., Downers Grove, Ill.). High activity samples were diluted with a Carbo-Sorb and Permafluor mixture as necessary. All samples were counted using high amplification to overcome the highly quenching characteristic of Carbo-Sorb. A standard curve of AES vs % efficiency was prepared using known DPM ^{14}C-toluene.

To determine fixation into soluble materials aliquots of the 80%

and 20% ethyl alcohol extracts were acidified with formic acid, and dried by nitrogen flow. The dried samples were then dissolved in 0.75 ml H_2O, 15 ml of PCS scintillation solution (Amersham-Searle, Arlington Heights, Ill.) was added, and the samples were counted by liquid scintillation. A standard curve of AES vs. % efficiency was prepared under these scintillation conditions. Data from both extracts were combined to give total fixation into soluble materials.

Results for $^{14}CO_2$ fixation into soluble, insoluble, and total products were expressed on the basis of microgram atoms of ^{14}C fixed/mg Chl. The microgram atoms ^{14}C were calculated by dividing the radioactive content of a product in μCi by the specific radioactivity of the entering $^{14}CO_2$ (16.6 μCi/μmole). In this way the μg atoms of ^{14}C in a product corresponds to a given number of μmoles of administered labeled carbon dioxide. Values determined in that way permit direct comparison between the overall rate of fixation and ^{14}C accumulation in different products (whether the product considered is a class of metabolites or an individual metabolite).

8. $^{14}CO_2$ Fixation Into Specific Soluble Metabolites. Aliquots of the 80% and 20% alcohol extracts were analyzed by several paper chromatography systems to obtain ^{14}C fixation into specific soluble metabolites. The systems used are described below. Whatman 1 chromatography paper was utilized. Results for the two extracts of each sample were combined. The fixation results were expressed on the basis of microgram atoms ^{14}C (calculated as described in section 7 above) fixed per mg Chl into each soluble metabolite.

Paper Chromatography System a. Most soluble metabolites were determined using a two-dimensional system essentially like that described by Pedersen et al. (1966b). Two chromatograms were prepared

from each extract: in one the chromatogram was developed for 24 hr in each direction (Fig. 8); in the other chromatogram was developed 48 hr in each direction (Fig. 9). In order to achieve good resolution, the preparation of the first dimension solvent for the 24 hr chromatograms was modified due to the acidic nature of the phenol reagent now used (Mallinckrodt Liquified Phenol A.R., approximately 88% phenol, 12% water). The phenol originally used by Pedersen et al. (1966b) was apparently not as acidic as the presently available material. The first dimension solvent for 24 hr chromatography was prepared by the following procedure (S. Krohne and G. Levine, unpublished): Mix thoroughly 7 liters of liquified phenol and 3 liters of water. Add 50 g Na_2CO_3 and stir overnight. Remove (by syphon) 8 liters of the resulting phenol phase. Mix a small aliquot of the phenol phase with an equal volume of distilled water. Determine the pH of the resulting water phase. It should be approximately pH 7. Add aliquots of glacial acetic acid to the phenol phase until the pH as determined above is in the range 4.1 - 4.4. Some 400 cc of glacial acetic acid is required. After pH adjustment add 1 ml of 1.0 M EDTA to each liter of final phenol solution. Use that solution as the first dimension 24 hr paper chromatography solvent. It will be referred to as the "treated phenol" solvent in the remainder of this thesis. The first dimension solvent for the 48 hr chromatography was prepared exactly as described by Pedersen et al. (1966b). It consisted of 840 ml liquified phenol (Mallinckrodt, 88% phenol, 12% H_2O), 160 ml of water, 10 ml glacial acetic acid, and 1 ml 1.0 M EDTA. This solvent will be referred to as the "untreated phenol" solvent in the remainder of this thesis. The second dimension solvent for both the 24 hr and 48 hr chromatograms was made up of equal volumes

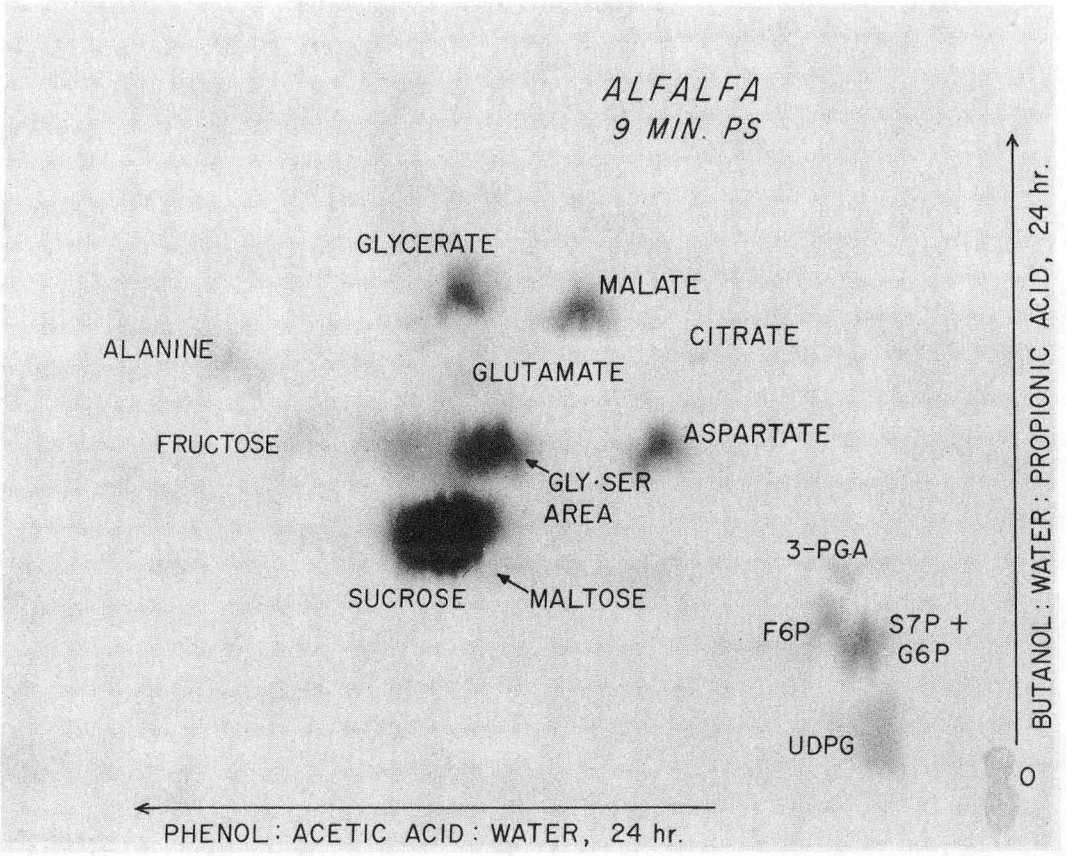

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Figure 8. Typical radioautograph of two-dimensional chromatogram prepared using paper chromatography system a. Alfalfa photosynthesis with 0.042% $^{14}CO_2$ in air (54.6 μCi/μmole) for 9 minutes. Extraction with 80% ethyl alcohol (v/v). See note in text on glutamate position and phenol used.

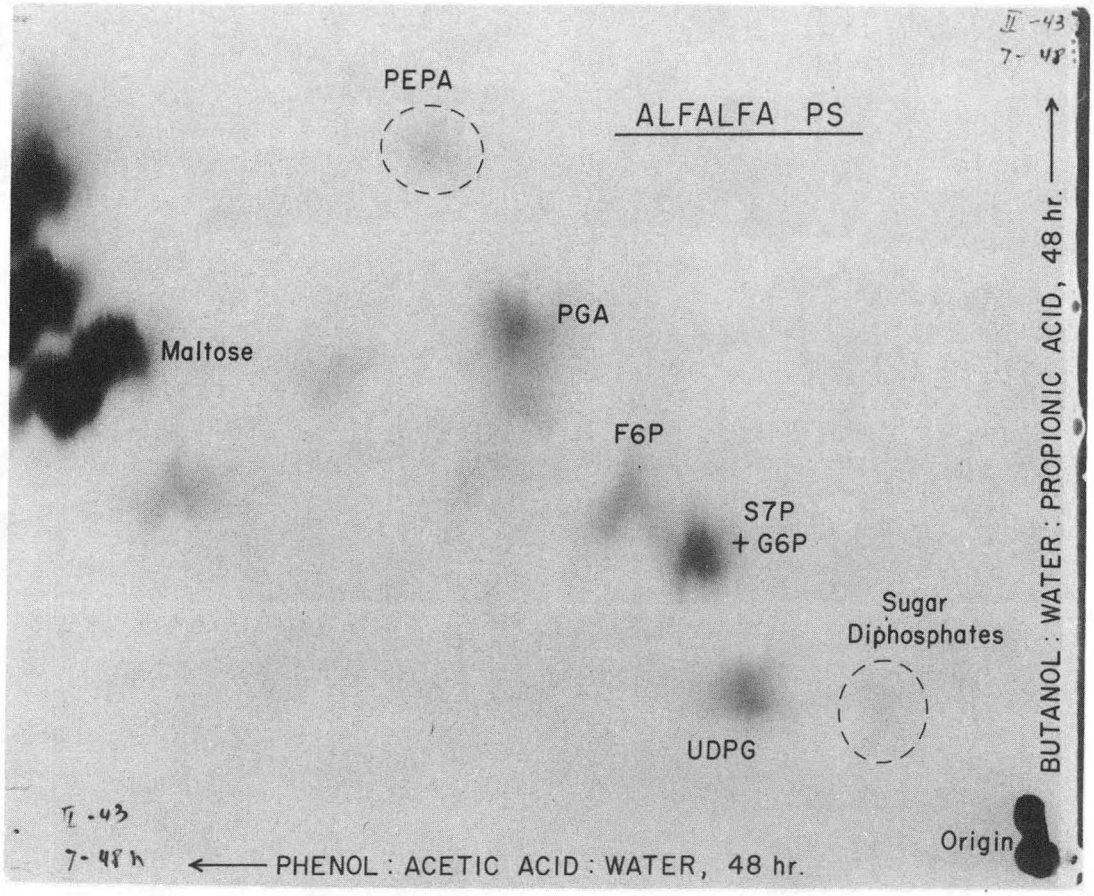

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Figure 9. Typical radioautograph of two-dimensional chromatogram prepared using paper chromatography system a. Alfalfa photosynthesis with 0.024% $^{14}CO_2$ in air (22.3 μCi/μmole) for 30 minutes. Extraction with 80% ethyl alcohol, 20% ethyl alcohol, and water. See note in text on phenol used.

of n-butanol-water (370:25 v/v) and of propionic acid-water (180:220 v/v).

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 with an automatic Geiger counter (Moses and Lonberg-Holm 1963). Between 85 and 95% of the soluble ^{14}C label applied to the chromatography papers was recovered in the radioactive areas located by radioautography. Major products formed during photosynthesis with $^{14}CO_2$ by alfalfa leaflets are shown in the radioautographs presented in Figs. 8 (24 hr chromatography) and 9 (48 hr chromatography). The chromatograph whose radioautograph is shown in Fig. 8 was obtained during a preliminary experiment. It was developed using "untreated phenol" solvent in the first dimension as the then available phenol was identical to that used by Pedersen et al. (1966b). Chromatograms prepared more recently using the "treated phenol" solvent were essentially similar with one significant change. The mobility of glutamate in the "treated phenol" dimension is just less than that of serine. Glutamate appeared to the right of the Gly•Ser area shown in Fig. 8. The 24 hr chromatograms were generally used to obtain data on most of the secondary products of photosynthesis (such as amino acids, sugars, organic acids). Data on fixation into RPP cycle intermediates (PGA, sugar phosphates) and other low chromatographic mobility compounds (such as PEPA and UDPG) was generally obtained from the 48 hr chromatograms. Glycolate, glycine, serine and glucose were not determined using these chromatograms. Glycolate is volatile in acidic solvents. Glycine, serine and glucose overlap on these chromatograms.

Paper Chromatography System b. I attempted to isolate and quantify glycolate labeling in alfalfa using the two-dimensional

paper chromatography system described by Bassham and Kirk (1973). It utilized phenol-water-ammonia in the first dimension and n-propyl alcohol-ammonia-water in the second dimension. It was developed for use in analysis of glycolate produced by algae, and is a basic system so as to avoid glycolate volatility problems. The first dimension solvent was made up of 754 ml aqueous phenol (88% phenol, 12% water, Mallinckrodt, A. R., not treated to adjust pH), 224 ml H_2O, 22.4 ml of concentrated NH_4OH, and 2 ml of 0.5 M EDTA. The second solvent was made up of 600 ml of n-propanol, 300 ml of concentrated NH_4OH, and 100 ml of H_2O. Due to the production of substantial amounts of labeled sucrose and glycerate in the alfalfa experiments, materials which overlapped the glycolate spot, this system proved unsuitable for glycolate determination. Radioactive products were located by radioautography. Fig. 10 shows the pattern obtained on the chromatograms of labeled alfalfa extract when this chromatography system was used. Metabolite locations shown (except for glycolate) were determined using nonlabeled carrier added to the labeled alfalfa extract. Glycolate location was determined using authentic ^{14}C-glycolate (Amersham-Searle) added to the alfalfa extract and also chromatographed separately.

Paper Chromatography System c. Glycolate labeling was determined using a newly developed basic two-dimensional chromatography system. That system was constructed from two previously described chromatographic solvents. The first dimension was the phenol-water-ammonia solvent prepared as described above for paper chromatography system b (Bassham and Kirk 1973). The second dimension consisted of n-butyl alcohol-95% ethyl alcohol-water-diethylamine (80:10:20:1) as described by Zelitch (1965). Chromatography was conducted for 32 hr in the first dimension followed by 16 hr drying. Chromatography was

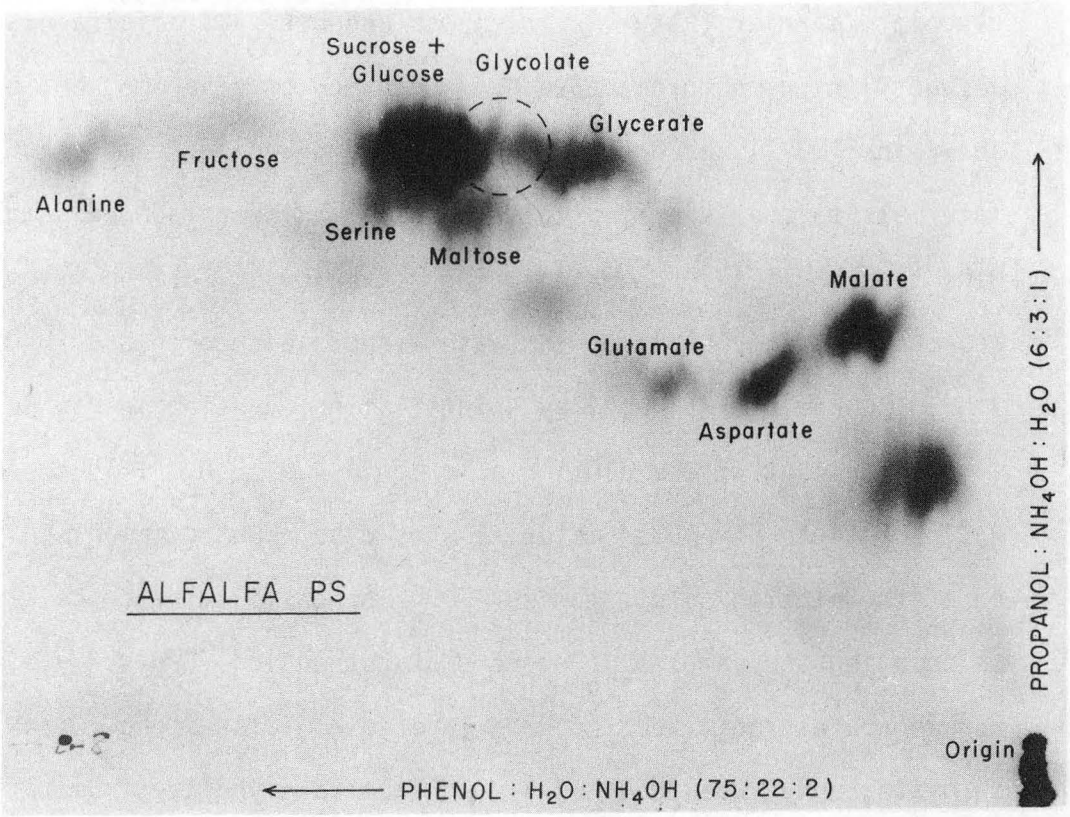

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Figure 10. Typical radioautograph of two-dimensional chromatogram prepared using paper chromatography system b. Alfalfa photosynthesis with 0.035% $^{14}CO_2$ (22.3 μCi/μmole) in air for 35 minutes. Extraction was as described in Fig. 9. Chromatography was for 30 hr in the PhOH direction, 24 hr in the propanol direction. See note in text on phenol used.

then conducted for 55 hr in the second dimension followed by 3.5 hr drying. Labeled products were located by radioautography (7 day exposure). A typical radioautograph is shown in Fig. 11. Glycolate location was determined by chromatography of authentic ^{14}C-glycolate separately, and also mixed into labeled alfalfa extract. Unlabeled glycolate carrier (about 1 μmole) was added to all alfalfa extract aliquots prior to chromatography. Location of other metabolites shown in Fig. 11 was determined by adding unlabeled samples of those materials to the labeled alfalfa extracts. The unlabeled samples were then chemically detected. For convenience, labeled glycolate was counted after elution with water (spots eluted in 3 cc H_2O, 30 C, 2 hr, 150 rpm shaker bath) by scintillation counting in Aquasol 2 (1.0 cc aliquot of the elution solution + 4.0 cc H_2O + 15 cc Aquasol 2). Solutions of eluted material should be counted immediately. The pH of the elution solution was 7-7.5. No chemiluminescence was observed on counting the samples. Recovery of authentic ^{14}C-glycolate, whether run separately or mixed into the labeled alfalfa extract prior to chromatography, was 89-94%. Eluted labeled glycolate from alfalfa extract was examined for purity by 22 hr paper chromatography using n-propyl alcohol-concentrated ammonium hydroxide-water (6:3:1). It was found to be free of material with other than the mobility of authentic ^{14}C-glycolate (Amersham-Searle).

Paper Chromatography System d. Glycine and serine did not separate well in chromatography system a. The glycine-serine area shown in Fig. 8 consisted of overlapped glycine, serine, and glucose spots. Glycine was found predominantly in the left side of that area, while serine and glucose were found predominantly in the right side. A new two-dimensional chromatography system was constructed for use

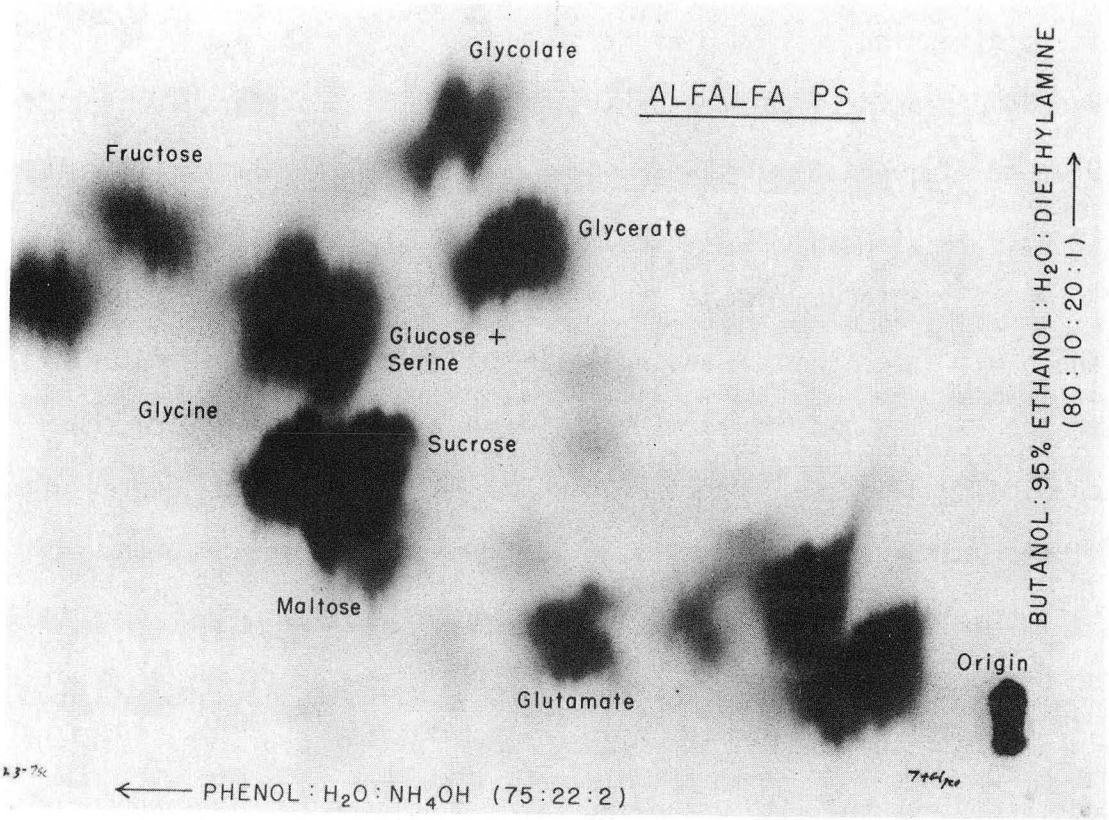

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Figure 11. Typical radioautograph of two-dimensional chromatogram prepared using paper chromatography system c. Alfalfa photosynthesis with $^{14}CO_2$ (25.8 μCi/μmole) in air. 150,000 DPM of labeled glycolate and 1 μmole of unlabeled glycolate added prior to chromatography. Extraction was as described in Fig. 9. Chromatography was for 32 hr in the phenol direction, 55 hr in the butanol direction. See note in text on phenol used.

in analysis of the glycine and serine content of the alfalfa extracts. The first dimension utilized the "treated phenol" solvent of chromatography system a. The second dimension solvent consisted of ethyl acetate - 85% formic acid - water (7:2:1), a solvent described by Fink et al. (1963). Chromatography was conducted for 30 hr in the first dimension, followed after drying by 7.8 hr chromatography in the second dimension. Unlabeled glycine and serine carrier, about 1.3 and 0.4 µmoles respectively, was added to all alfalfa extract aliquots prior to chromatography. Labeled products were located by radioautography. A typical radioautogram is shown in Fig. 12. Location of metabolites shown was determined by adding unlabeled samples of those materials to the labeled alfalfa extracts. The unlabeled samples were then chemically detected. Glycine, glucose, and serine were counted after elution with water (5 cc H_2O, 30 C, 1 hr, 150 rpm shaker bath, elution recovery 100%) by scintillation counting in Aquasol 2 (0.5 cc elution solution + 4.5 cc H_2O + 15 cc Aquasol 2). The identities of the eluted labeled glycine, serine and glucose were further confirmed by 21 hr co-chromatography with unlabeled carrier using n-butanol-propionic acid-water solvent (Pedersen et al. 1966b).

9. Chl Extraction and Determination. Bruinsma's (1963) Chl extraction procedure calls for the use of 80% acetone as solvent while extraction of photosynthetic metabolites is better accomplished with 80% ethyl alcohol. Comparison Chl determinations were made with opposite leaflet halves extracted with those two solvents. Comparisons were also made between two 80% ethyl alcohol Chl extraction procedures used in my alfalfa work, again using opposite leaflet halves. The effect of adding ammonium hydroxide to the Chl extraction solvent was examined. Ammonium hydroxide prevents pheophytin formation during

extraction (Strain and Svec 1966). For each extraction experiment, three leaflets of a single leaf were cut in half along the leaflet midrib and divided into two equivalent sets (a and b). The fresh weights of the sets were determined. One set was then extracted using each of the two procedures to be compared. In all cases leaflets were first ground at liquid nitrogen temperature using the tissue grinding equipment described earlier. Additional grinding at dry ice-acetone temperature with 5 ml of solvent (Table 2) followed. Ground leaflet suspensions were stirred magnetically in the dark for the amounts of time and at the temperatures indicated in Table 2. Suspensions were centrifuged (IEC centrifuge) for 3 min at 2500 rpm. The supernatant solutions were diluted as necessary and their visible absorption spectra determined. Spectra were corrected for light scattering. The Chl content of each set (μg Chl/mg fresh weight) was calculated using the equations given by Bruinsma (1963). The extraction solvents and conditions investigated are presented in Table 2. The Chl content determine for each leaflet set is reported in Table 3. Each value reported is the average of the values determined by Bruinsma's two equations.

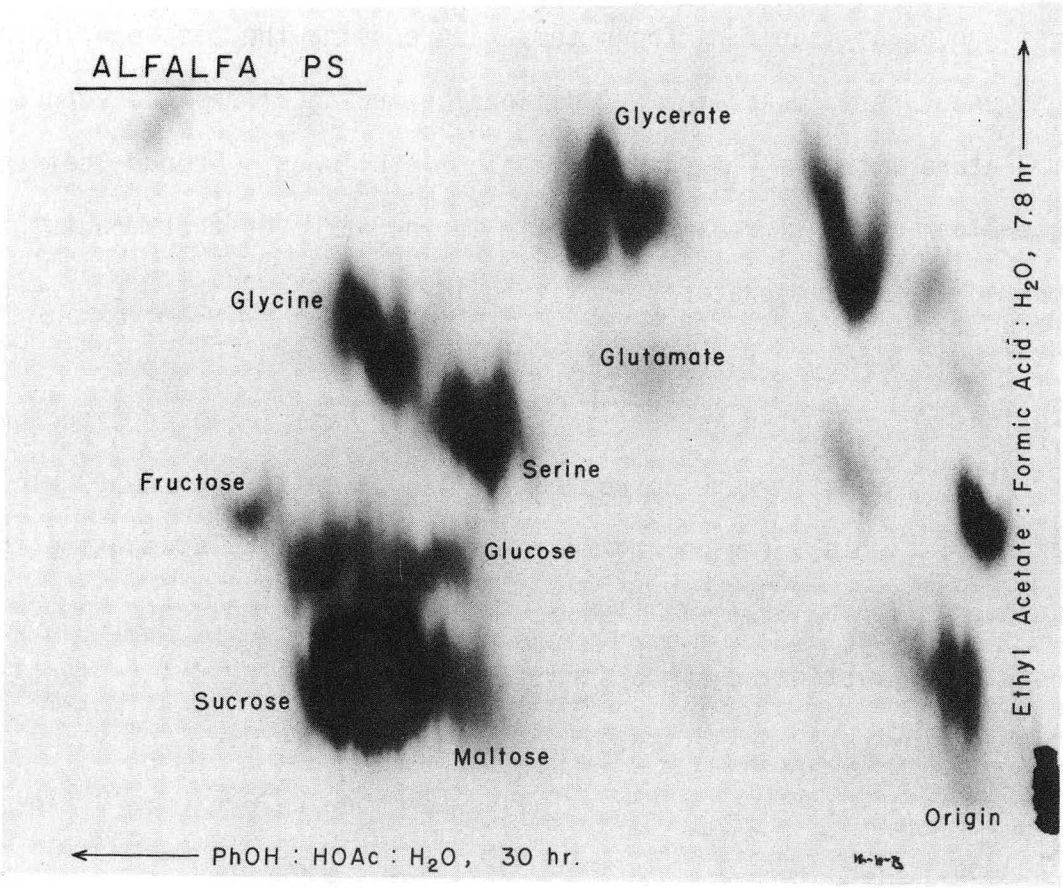

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Figure 12. Typical radioautograph of two-dimensional chromatogram prepared using paper chromatography system d. Alfalfa photosynthesis with 0.026% $^{14}CO_2$ (25.8 μCi/μmole) in air for 22 min. 1.3 μmoles unlabeled glycine and 0.4 μmoles unlabeled serine added prior to chromatography. Extraction was as described in Fig. 9. See note in text on phenol used.

Table 2. Chl Extraction Procedures Investigated

Procedure	Extraction Solvent (v/v)	Extraction Time (hr)	Extraction Temperature (C)
A	80% Acetone	2.5	0
B	80% Ethyl Alcohol	2.5	0
C	80% Ethyl Alcohol	1.5	20
D	80% Ethyl Alcohol (several drops ammonium hydroxide added to leaflets prior to grinding)	1.5	20

Table 3. Chl Content Determined for Leaflet Sets*

Experiment	Extraction Procedure Used (see Table 2)		Chl Content (µg Chl/mg fresh weight)		Percent Difference Between Sets
	Leaflet Set a	Leaflet Set b	Set a	Set b	
1	B	B	1.99	2.03	2
2	B	B	2.14	2.05	4
3	A	B	1.79	1.86	4
4	A	B	1.87	1.80	4
5	B	C	1.65	1.65	0
6	B	C	1.69	1.60	5
7	C	D	2.23	2.11	5
8	C	D	1.64	1.62	1

* Sets of opposite alfalfa leaflet halves were extracted (Table 2) and their Chl content determined as described in the text.

Experiments 1 and 2 (Table 3) indicate that the comparison of extraction procedures using opposite leaflet halves is a valid approach. Experiments 3 and 4 (Table 3) indicate that the two solvents utilized give equivalent results as to leaflet Chl content. Bruinsma's (1963) coefficients can still be used when 80% ethyl alcohol is substituted for 80% acetone. (It can be noted that the peak maxima of the Chl spectra in the 660 nm region was 665 nm in 80% ethyl alcohol, while it was 663 nm in 80% acetone. As apparent in Table 3, this difference did not result in a significant change in calculated leaflet Chl content.) Experiments 5 and 6 (Table 3) indicate that the two ethyl alcohol extraction procedures give equivalent results. Experiments 7 and 8 indicate that it is not necessary to add base to the leaflets prior to grinding. Chl content calculated for a given leaflet set using Bruinsma's two equations generally did not differ by more than 1-5%; this is indicative of the absence of significant Chl degradation during the extraction procedures examined (Bruinsma 1963).

10. Photosynthetic Rate Conversion Factor. Sets of three central alfalfa leaflets were selected as described for the $^{14}CO_2$ labeling experiment. The total leaflet area of each set was measured. The leaflet sets were extracted by procedure C (Table 2) and their Chl content determined (Bruinsma 1963). An average Chl content of 3.2 mg Chl/dm^2 was found. A similar value has been found for tobacco (Zelitch 1971). Based on that factor, alfalfa photosynthetic rates in this thesis, which are given in units of μmoles CO_2/hr•mg Chl, can be divided by 7 to give approximate CO_2 fixation rates in units of mg CO_2/hr•dm^2. That unit is often used in physiologically oriented plant research.

C. Results

The rate of photosynthesis measured by means of the $^{12}CO_2$ analyzer during a portion of the $^{12}CO_2$ part of the experiment was 90 µmoles CO_2/hr•mg Chl at 0.033% CO_2. The data obtained for total ^{14}C incorporation into the leaflets is shown in Fig. 13. The total ^{14}C incorporation plot indicates that 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 expected due to the slightly lower carbon dioxide concentration (Gaastra 1959).

The data obtained for ^{14}C incorporation into soluble products and incorporation into insoluble products are shown in Figs. 13 and 14. Figure 15 presents data for fixation of ^{14}C into soluble and insoluble products as a percent of the total labeled carbon fixed at each sample time. Labeled carbon first flowed into pools of soluble materials and it was only after several minutes of photosynthesis that it began to be incorporated into insoluble products (probably polysaccharides, proteins, etc.). As some of the active soluble pools saturated with ^{14}C (see later discussion) the tracer carbon flow into insoluble products increased to approximately 55% of the total.

The labeled products observed on the two-dimensional paper chromatograms (Figs. 8-12) were generally similar to those reported previously in other higher plant species (Tamas and Bidwell 1970, Jensen and Bassham 1966). In Fig. 16 are shown the labeling curves of several types of products identified. Included are data on an amino acid (alanine), a carbohydrate synthesis intermediate (UDPG), a tricarboxylic acid cycle intermediate (citrate), an RPP cycle intermediate (PGA),

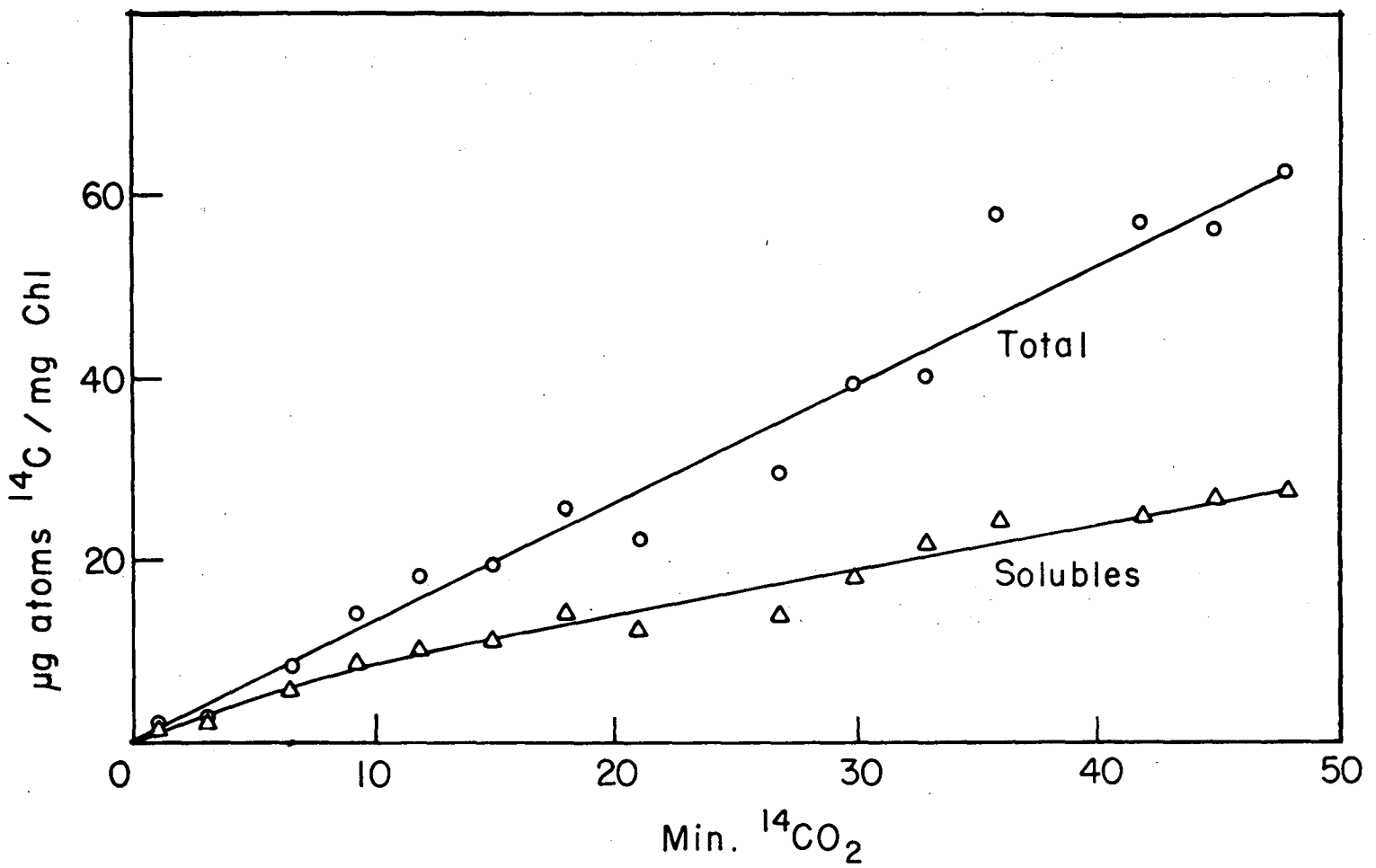

Figure 13. Total photosynthetic ^{14}C incorporation, and ^{14}C incorporation into 80% and 20% ethyl alcohol-soluble products, by alfalfa leaflets exposed to 0.027% $^{14}CO_2$ (16.6 μCi/μmole) in air (20% O_2) at 3600 ft-c, and at 18 C.

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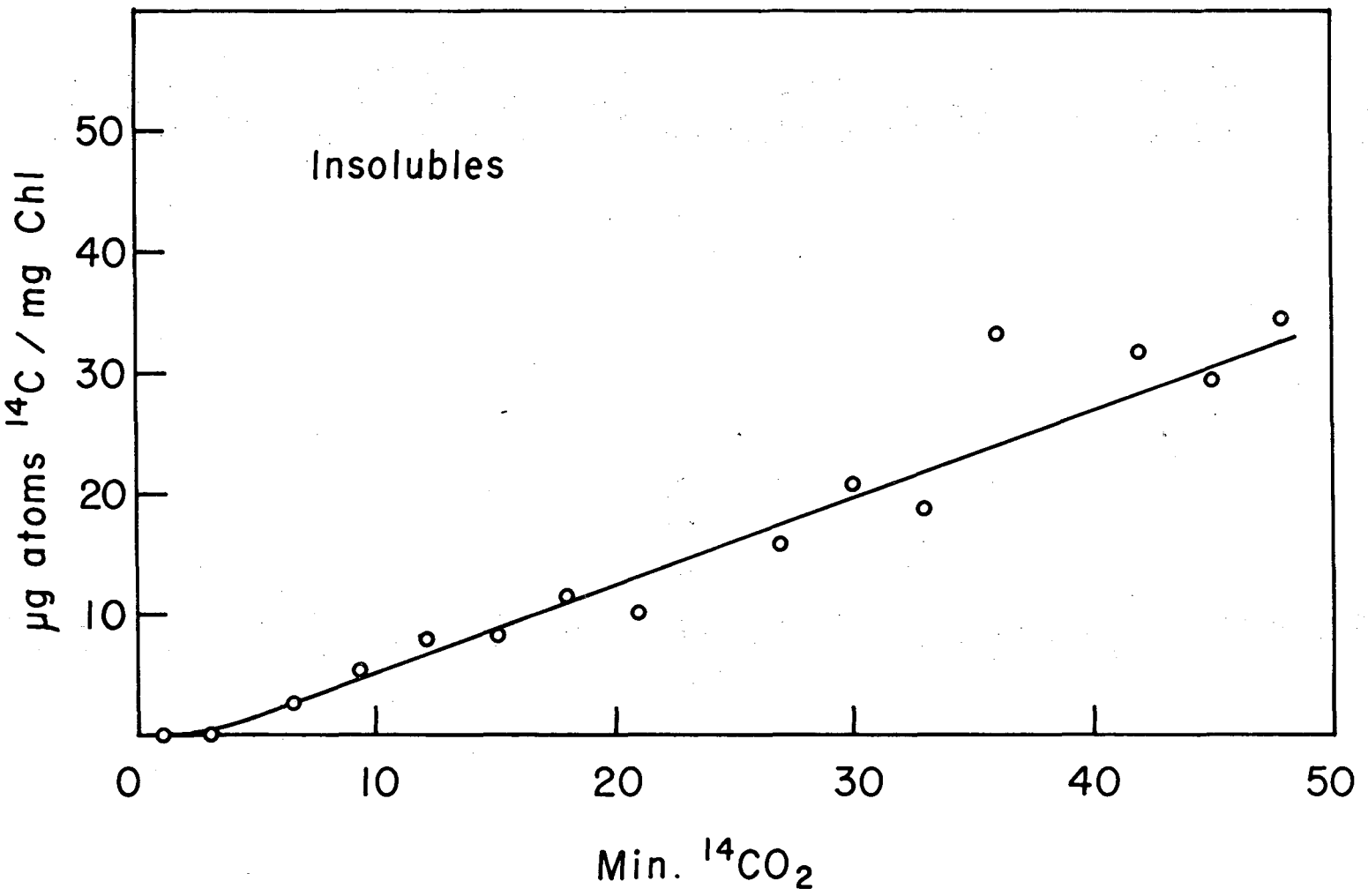

Figure 14. Photosynthetic ^{14}C incorporation into 80% and 20% ethyl alcohol-insoluble products, by alfalfa leaflets exposed to $^{14}CO_2$ as described in Fig. 13.

XBL7511-8763

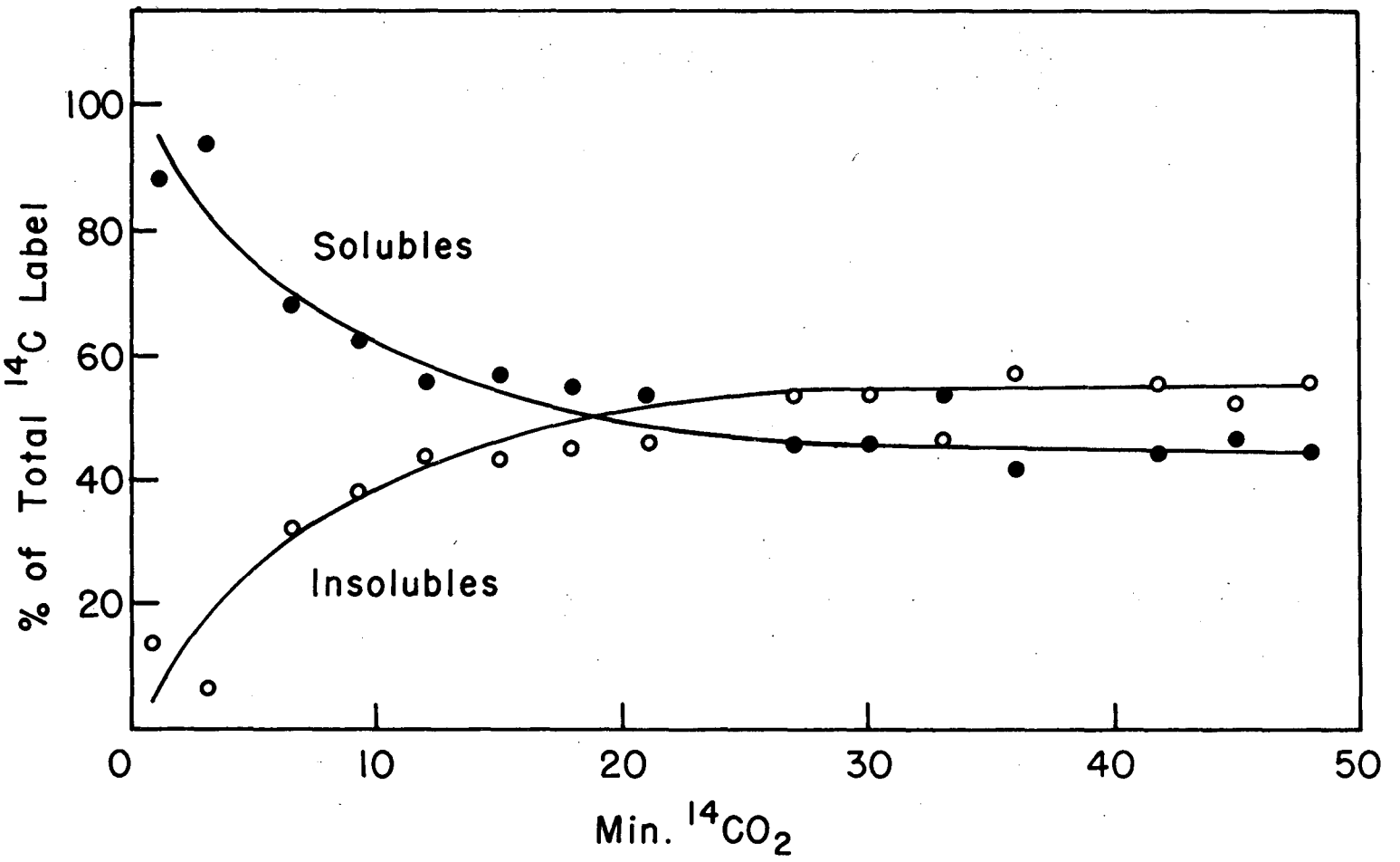

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Figure 15. Photosynthetic ^{14}C incorporation into 80% and 20% ethyl alcohol-soluble and insoluble products, by alfalfa leaflets exposed to $^{14}CO_2$ as described in Fig. 13.

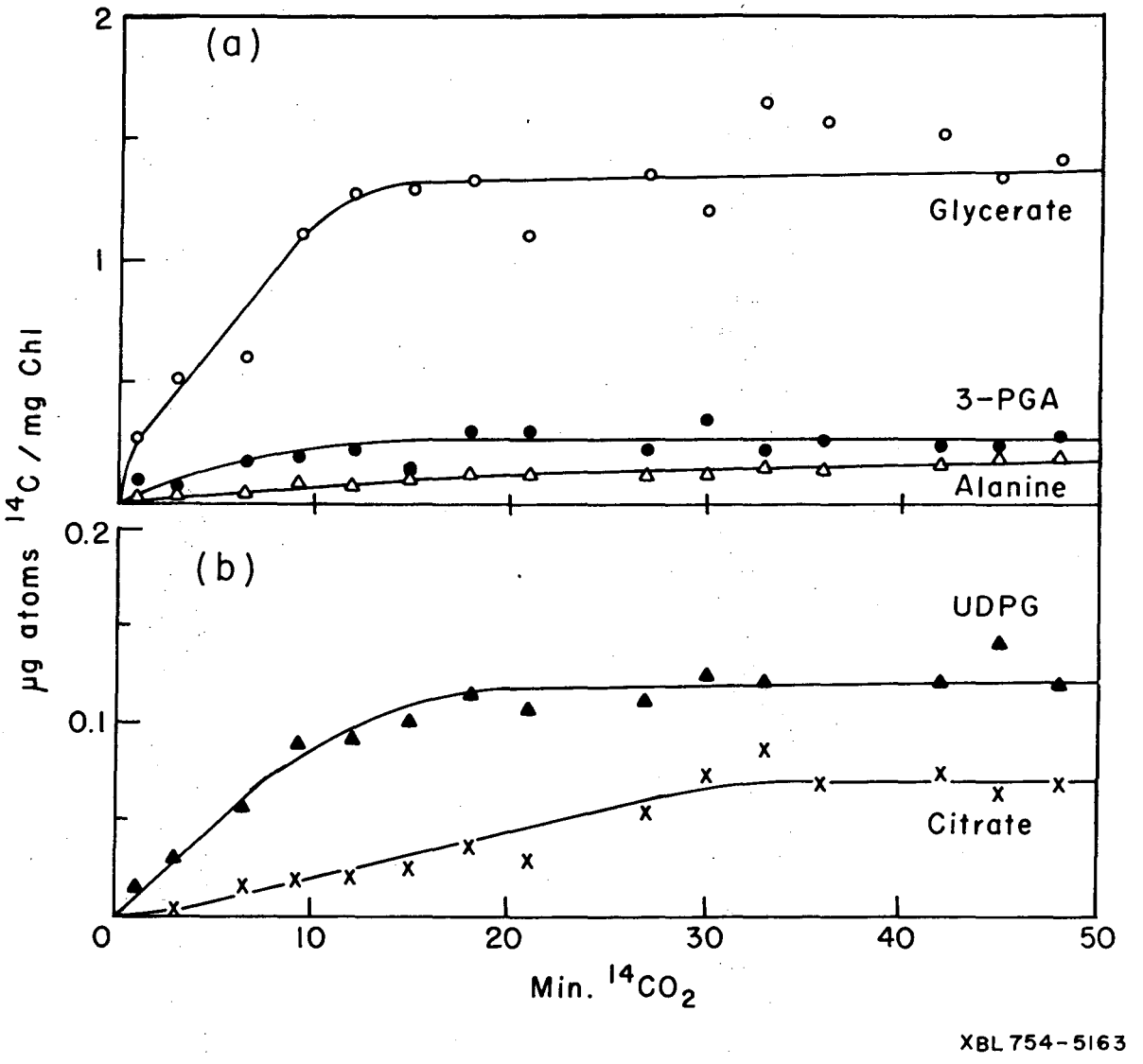

Figure 16. Incorporation of ^{14}C into several metabolites by alfalfa leaflets exposed to $^{14}CO_2$, and extracted, as described in Fig. 13.

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 early metabolite pools during steady state photosynthesis in algae (Bassham 1973, Bassham and Kirk 1960). When photosynthesis with tracer carbon dioxide is initiated following a preliminary period of photosynthesis under the same conditions but with $^{12}CO_2$, the concentration of labeled material in such active metabolic pools rises until they are saturated with ^{14}C. From that time on the specific radioactivity of the material in those pools is identical to that of the incoming $^{14}CO_2$ (neglecting possible dilution from endogenous carbon sources). The saturation value level of label in each metabolite is then a direct measure of the amount of material in each active metabolite pool. Saturation of the pools of PGA, UDPG, and glycerate was reached within 10 to 20 min of photosynthesis with $^{14}CO_2$. The labeling curves of PGA and glycerate are especially similar. Citrate reached apparent saturation somewhat later (30 min) as might be expected for a substance that is quite removed from the $^{14}CO_2$ entry point. Other pools which reached saturation during the experiment were maltose (Fig. 17), sugar mono and diphosphates (Figs. 18 and 19), malate (Fig. 18), PEPA (Fig. 19), and glutamate (Fig. 20). Aspartate data was somewhat scattered but indicated the achievement of a steady state saturation level of approximately 0.24 μmole ^{14}C/mg Chl after about 30 min of photosynthesis with $^{14}CO_2$. Alanine labeling (Fig. 16) increased more rapidly at first, and then at a lower rate as saturation was approached. This may be indicative of some flow of endogenous unlabeled carbon into the alanine pool, or of the existence of two slowly interacting pools of alanine in the leaflets.

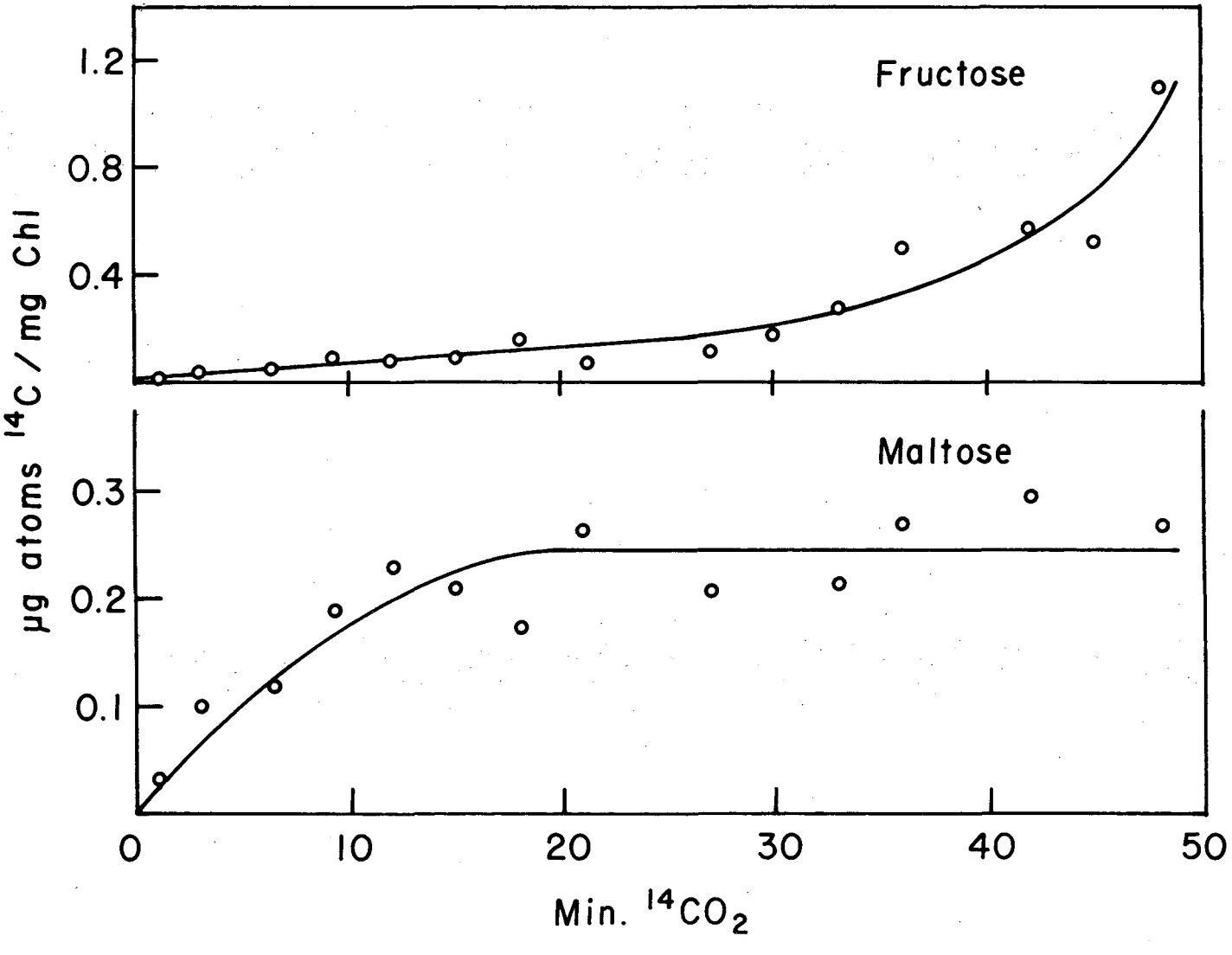

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Figure 17. Incorporation of ^{14}C into fructose and maltose by alfalfa leaflets exposed to $^{14}CO_2$, and extracted, as described in Fig. 13.

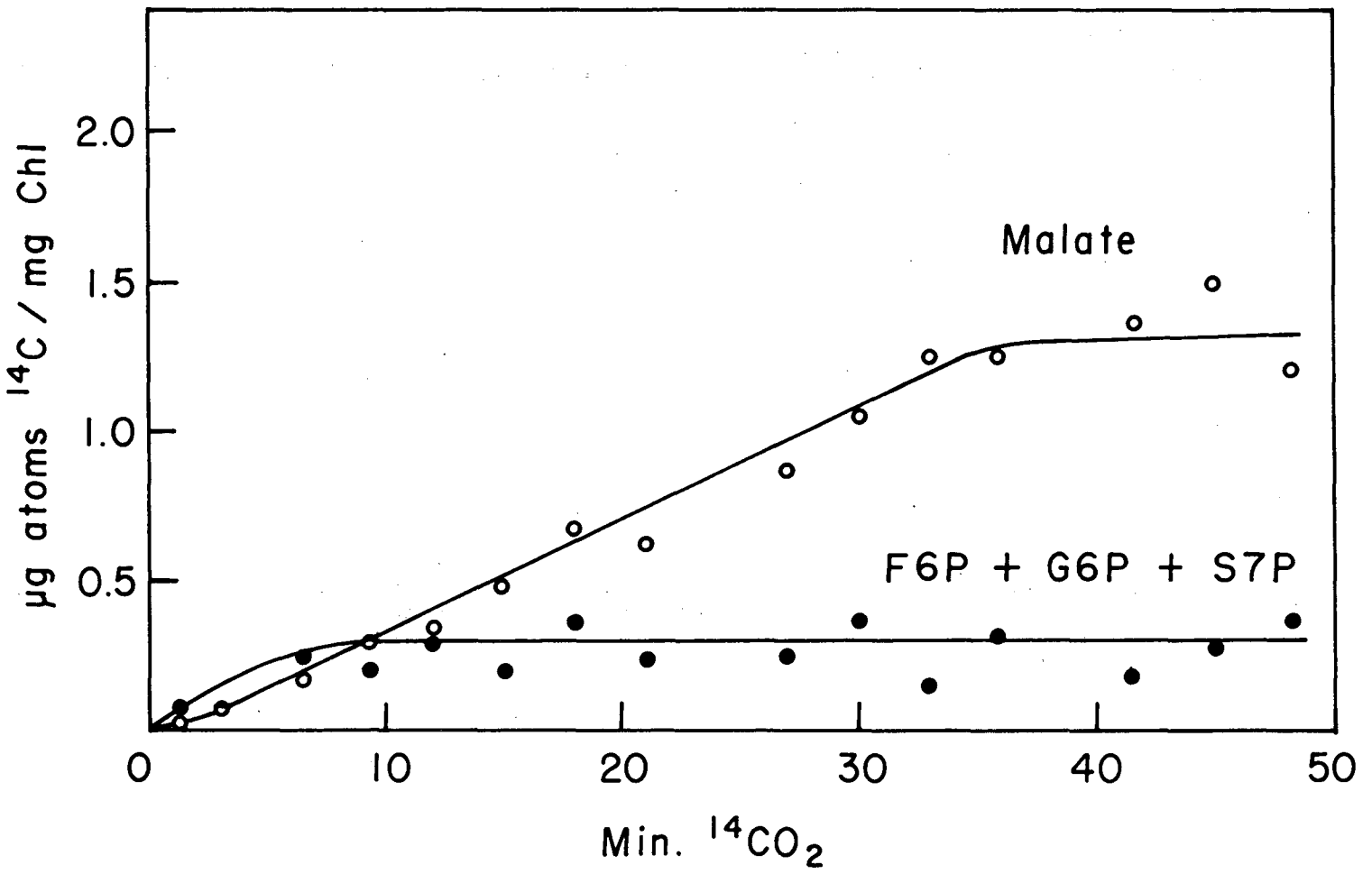

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Figure 18. Incorporation of ^{14}C into malate and the sugar monophosphates by alfalfa leaflets exposed to $^{14}CO_2$, and extracted, as described in Fig. 13.

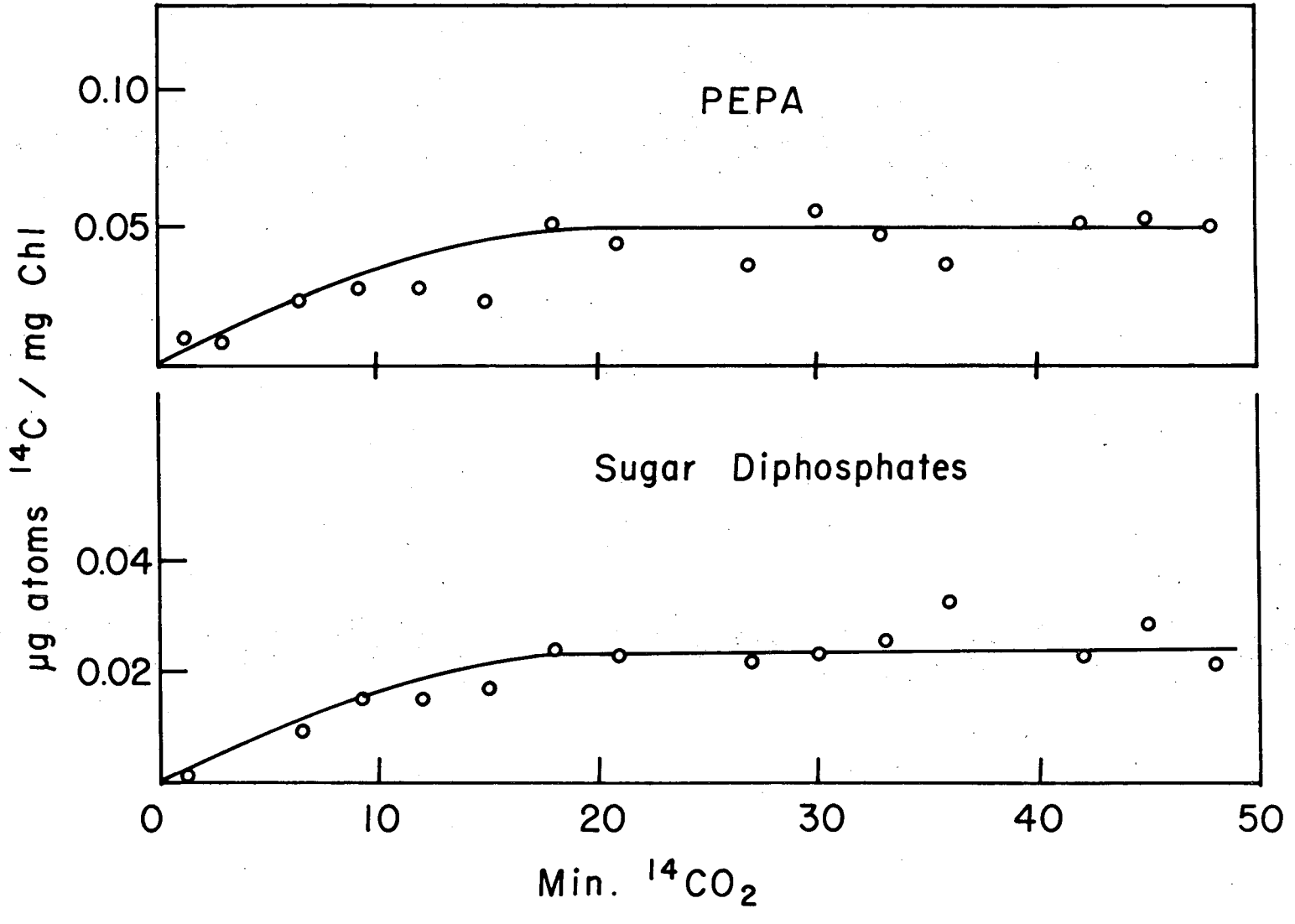

Figure 19. Incorporation of ^{14}C into PEPA and the sugar diphosphates by alfalfa leaflets exposed to $^{14}CO_2$ and extracted, as described in Fig. 13.

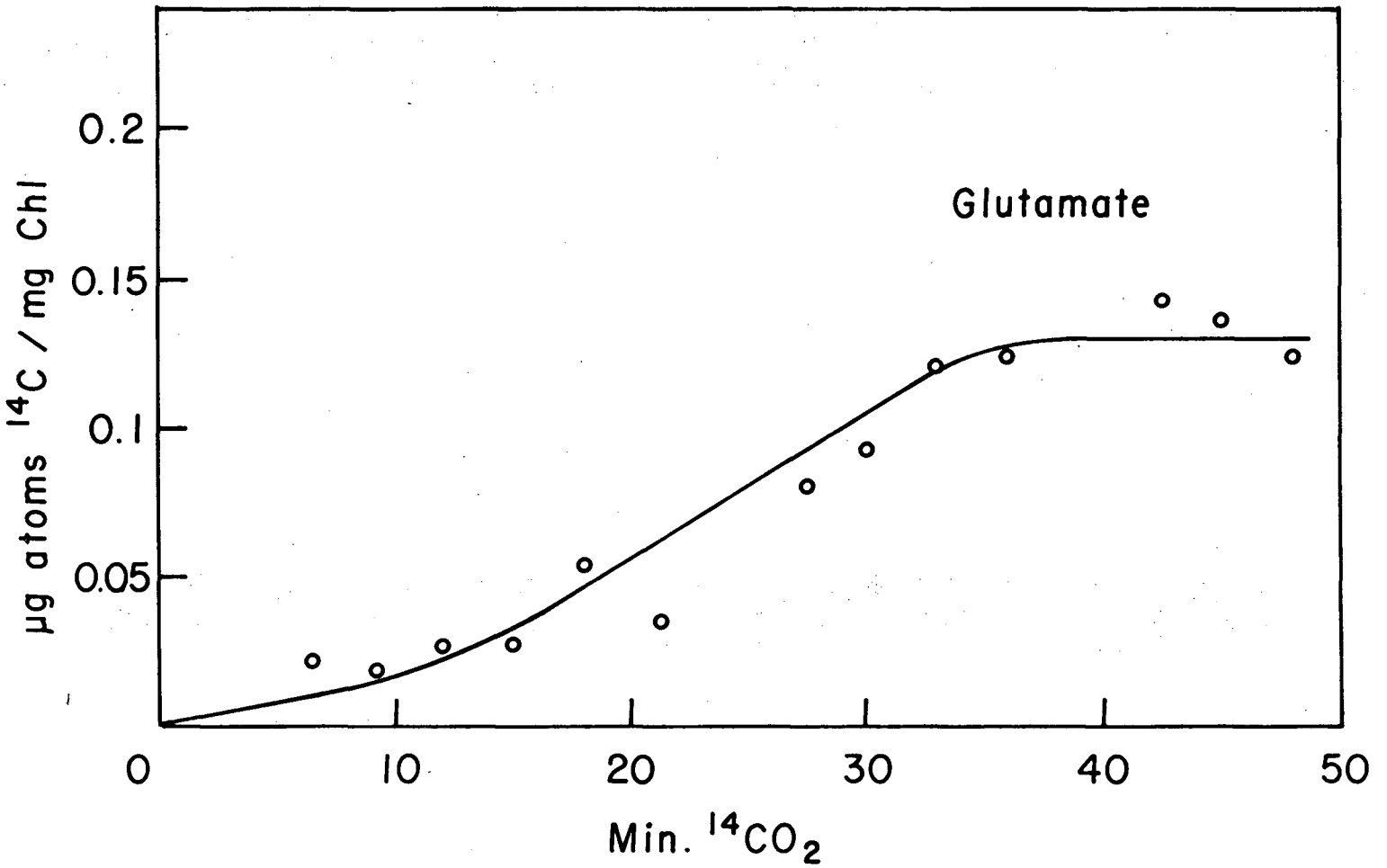

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Figure 20. Incorporation of ^{14}C into glutamate by alfalfa leaflets exposed to $^{14}CO_2$, and extracted, as described in Fig. 13.

Label content of the sucrose pool did not reach saturation (Fig. 21) while the pools of its probable precursors UDPG and F6P (Zelitch 1971) apparently reached saturation quite rapidly (Figs. 16 and 18). Sucrose ^{14}C content increased linearly with time after a short lag during which its precursors became labeled with ^{14}C. After the first three minutes of photosynthesis with $^{14}CO_2$, 7% of the soluble label was found in the sucrose pool. After forty-two minutes of photosynthesis 37% of the soluble ^{14}C was found in the sucrose pool. Sucrose was then by far the major labeled soluble metabolite formed.

Incorporation of ^{14}C into the area designated the Gly•Ser area in Fig. 8 was approximately linear with time, and reached a value of 5 μmoles ^{14}C/mg Chl after 40 min of photosynthesis. Glycine, serine, and glucose overlapped in the chromatography system used to obtain the radioautograph shown in Fig. 8. Those materials were successfully resolved using chromatography system d (Fig. 12). Using that system the data on fixation into glycine, serine, and glucose shown in Fig. 22 was obtained. ^{14}C incorporation into those materials was substantial. After only one minute of photosynthesis, approximately 5% of the total soluble ^{14}C was accounted for as glycine and 10% as serine. The fraction of soluble ^{14}C found in those pools then remained approximately constant as photosynthesis continued for an additional 47 min. Glycolate labeling is also shown in Fig. 22. The general shapes of the glycolate and glycine labeling curves are similar. The label content of each of those pools rose for about 35 minutes and then showed an indication of reaching saturation. Saturation was not approached until some 25 minutes after the pools of glycerate and PGA had already attained it (Fig. 16).

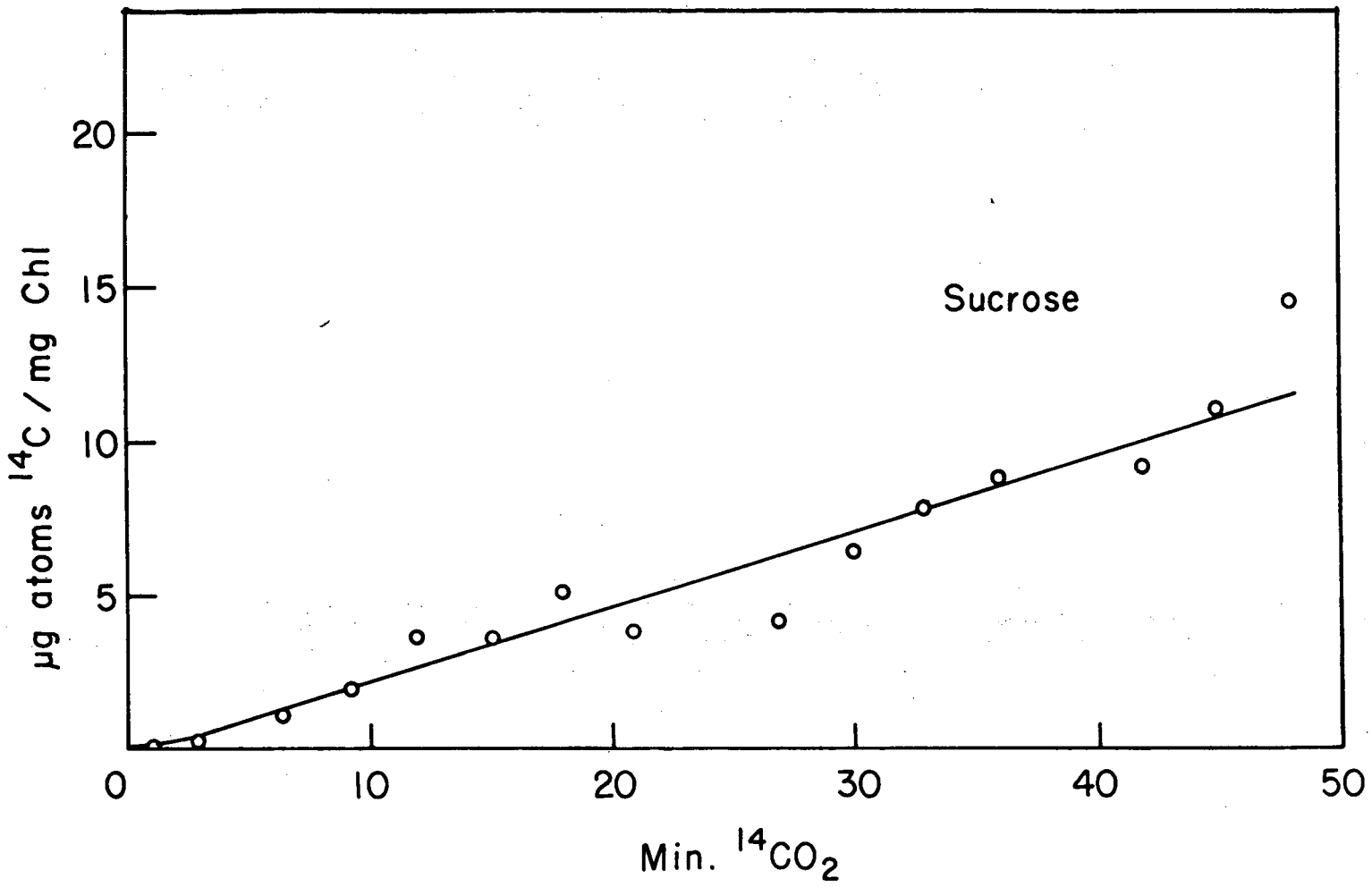

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Figure 21. Incorporation of ^{14}C into sucrose by alfalfa leaflets exposed to $^{14}CO_2$, and extracted, as described in Fig. 13.

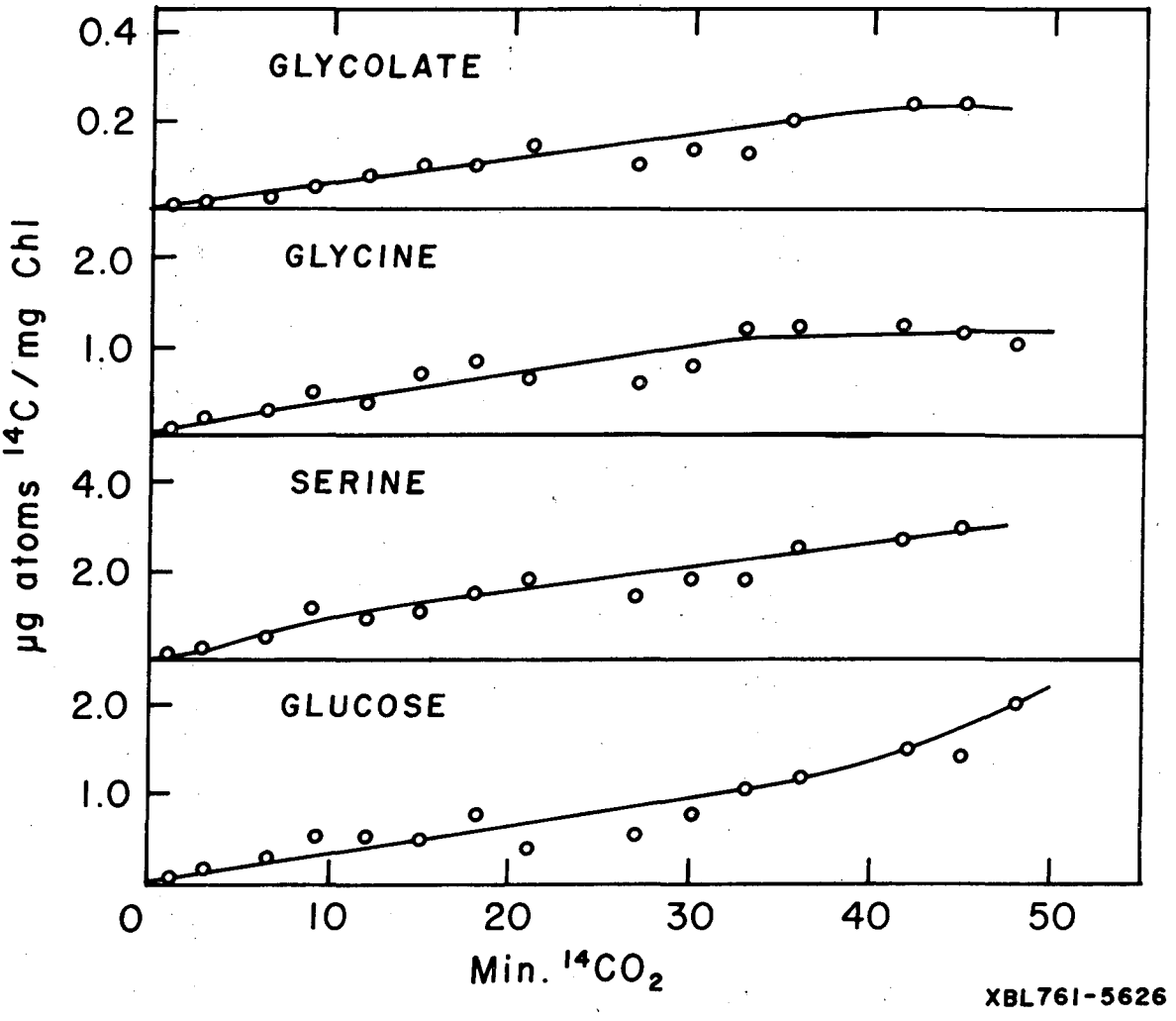

Figure 22. Incorporation of ^{14}C into several metabolites by alfalfa leaflets exposed to $^{14}CO_2$, and extracted, as described in Fig. 13.

In addition to the free sugars sucrose, glucose, and maltose, a sizeable amount of free fructose was produced during photosynthesis with $^{14}CO_2$ (Fig. 17). The free glucose and fructose may have arisen to some extent from in vivo action of invertase on the large pool of sucrose produced. As the maltose pool (Fig. 17) reached early saturation with ^{14}C (20 min) it is unlikely that it has arisen from starch hydrolysis during leaf extraction, but rather plays some metabolic role in alfalfa.

D. Discussion

The photosynthetic rate measured at atmospheric CO_2 concentration was 90 μmoles/hr•mg Chl. That can be converted to a value of 13 mg CO_2/hr•dm^2 using the factor given earlier (Materials and Methods). That rate is comparable to values previously reported in the literature for alfalfa photosynthesis. For example, Wolf and Blazer (1972) reported light saturated photosynthetic rates of attached alfalfa leaves (var. Stride) ranging from 3 to 15 mg CO_2/hr•dm^2 depending directly on the light level at which the plants were grown. Pearce et al. (1968) reported photosynthetic rates measured on excised alfalfa leaves (var. Williamsburg) generally in the range of 20 to 40 mg CO_2/hr•dm^2 for leaves grown in a growth chamber. Delaney et al. (1974) reported rates of 9-29 mg CO_2/hr•dm^2 for leaves harvested from plants grown under field conditions. Evidently the excised alfalfa leaflets I used in my experiments photosynthesized at a reasonable rate in my leaf exposure device. Saturation of many metabolite pools with ^{14}C (see for example Fig. 16) occurred while photosynthesis was continuing at a constant rate. This is proof that the leaf exposure system allows for the establishment of a steady state and makes possible the study of steady

state photosynthesis by whole alfalfa leaflets. It was expected that some metabolites would not reach saturation during the experiment due to the limited time allowed for photosynthesis with $^{14}CO_2$. The level of label found in sucrose did not reach a constant value (Fig. 21) even though its probable major precursors F6P and UDPG apparently saturated rapidly (Figs. 16 and 18). The high level of sucrose formation observed is in accord with nonkinetic data presented by Norris et al. (1955) for other higher plant species. Apparently the large size of the sucrose pool, and possibly the end product nature of sucrose production in leaves of plants with nonphotosynthetic tissue, prevented the achievement of the type of saturation curves shown in Fig. 16.

Examination of the results from the entire set of alfalfa leaflet samples indicates that the active steady state pool sizes of the identified reductive pentose phosphate cycle intermediates (sugar mono and diphosphates, PGA) were small, relative to the total ^{14}C incorporation into pools of other soluble products. This is in accord with conclusions drawn about other species of higher plants (Jensen and Bassham 1966, Voskresenskaya et al. 1970). After twelve minutes of photosynthesis with $^{14}CO_2$ identified RPP cycle intermediates made up only 5% of the total soluble labeled products. The rapid appearance of label in other than RPP cycle pools (secondary metabolites such as sugars, TCA intermediates, amino acids) is indicative of the export of recently fixed carbon from the chloroplast and its immediate use in the synthesis of various intermediates (Smith et al. 1961, Heber 1974).

Tracer carbon flow from the RPP cycle to the tricarboxylic acid cycle appears to be occurring by the paths shown in Fig. 23 by the solid lines. The direct use of RPP cycle intermediates, without prior incorporation into sugar, in the synthesis of such secondary metabolites as

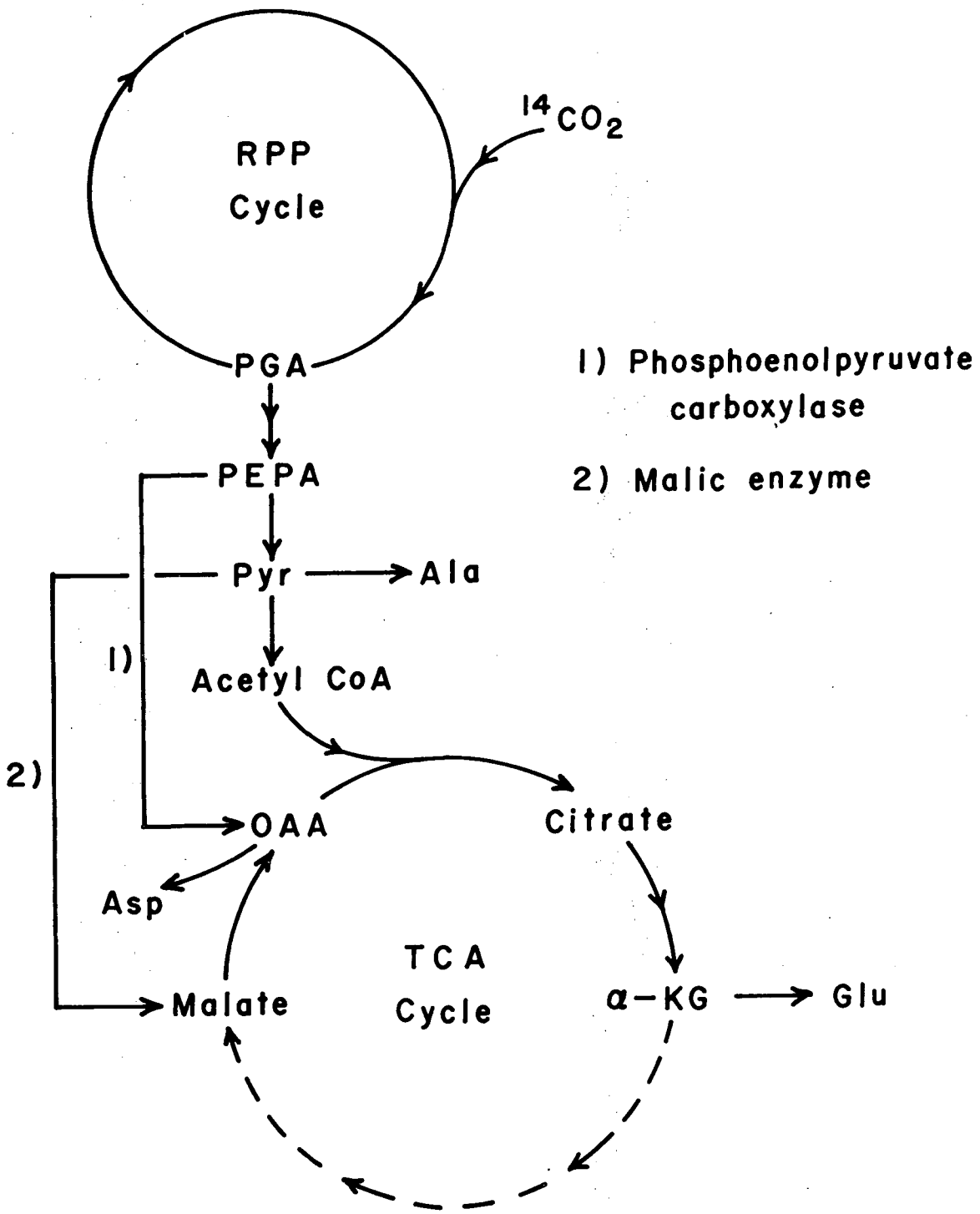

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Figure 23. Suggested paths of tracer carbon flow. Many steps, intermediate compounds and details are omitted from this diagram which is intended to show a general view of some metabolic connections.

amino acids was shown by Smith et al. (1961) and discussed by Bassham (1971). The role of PEP carboxylase in C_3 plants in the production of malate has been discussed recently by Ting and Osmond (1973a, b). The possible role of malic enzyme in plant tissue organic acid synthesis has been mentioned by Jacoby and Laties (1971) and earlier by Kanazawa et al. (1970). The extent of tracer carbon flow through acetyl CoA and around the TCA cycle during photosynthesis is presently a matter of controversy (Chollet and Ogren 1975). Hence a dotted line is used in Fig. 23 between α-KG and malate. Some researchers believe that the full TCA cycle does operate in the light (Zelitch 1971, Chapman and Graham 1974). Note that, in conformity with Fig. 23, the active pools of PGA, sugar mono and diphosphates, and PEPA (Figs. 16, 18, 19) were observed to reach saturation after 10-20 min of photosynthesis with $^{14}CO_2$, while citrate (Fig. 16), malate (Fig. 18) and glutamate (Fig. 20) reached saturation after approximately 35 min of photosynthesis with $^{14}CO_2$.

It is known that many plants, including alfalfa, contain 3-phosphoglycerate phosphatase (Randall et al. 1971). That enzyme may, under some conditions, carry out the conversion of 3-PGA to glyceric acid, eventually producing glycine and serine (Randall et al. 1971). It has been suggested that carbon flow in that direction may provide a means of substituting for or bypassing the glycolate pathway while still providing glycine and serine for biosynthesis (Randall et al. 1971, Tolbert 1973b). The glycolate pathway includes the sequence glycolate→→glycine→serine→→glycerate. The proposed alternative to that pathway, initiated by 3-phosphoglycerate phosphatase, is the sequence 3-PGA→glycerate→→serine→glycine. The alternative path involves some steps which are reversals of glycolate pathway reactions, but no synthesis of

glycolate is necessary to obtain glycine and serine.

The glycerate labeling curve was quite similar to that of 3-PGA. Label uptake into glycerate and 3-PGA (Fig. 16) reached saturation at about the same time. Glycerate became saturated well before glycolate, glycine and serine (Fig. 22). It seems reasonable to conclude that the synthesis of glycerate was occurring directly from 3-PGA by mediation of 3-phosphoglycerate phosphatase and not through the glycolate→→glycine→serine→glycerate sequence of the glycolate pathway.

The probable synthesis of glycerate directly from 3-PGA has been reported to occur in the leaves of several other species of plants (Rabson et al. 1962, Hess and Tolbert 1966, Vill and Parnik 1974). The formation of glycerate by the glycolate pathway has been shown to occur by precursor feeding experiments (Tolbert 1973a, Rabson et al. 1962).

Conditions resulting in predominant glyceric acid formation from PGA may coincide with those associated with low photorespiration. A substantial amount of data indicates that glycolate metabolism is the primary source of CO_2 in photorespiration. The immediate source of glycolate itself and the specific reaction mechanism by which most photorespiratory CO_2 is released are still in dispute (Zelitch 1971 and 1975a and b, Jackson and Volk 1970, Tolbert 1973a and b). However, the glycolate pathway is well accepted as existing in leaves and contributing to photorespiratory CO_2 release through the glycine→serine conversion (Tolbert 1973a and b, Zelitch 1975b). Conditions that repress photorespiration and therefore glycolate biosynthesis should decrease flow along the glycolate pathway from glycolate to glycerate. This could permit the alternative flow from 3-PGA to glycerate to dominate. It is possible that the low temperature (18 C) at which the

alfalfa experiment was conducted somewhat depressed photorespiration (Zelitch 1971, Jackson and Volk 1970) and favored predominant glycerate synthesis from 3-PGA. However, glycerate synthesis was still substantial and cannot be said to result from the operation of a residual minor metabolic path. After 12 min of photosynthesis with $^{14}CO_2$, 12% of the soluble label was accounted for as glycerate. PGA→glycerate flow appears to be a major metabolic path in alfalfa.

The data presented in this chapter did not indicate whether glycine and serine were synthesized from glycolate (Tolbert 1973a) or from 3-PGA (Randall et al. 1971). Further experiments will be necessary to determine the source of those two amino acids. It can be noted, however, that the amount of labeled glycine and serine produced was high and comparable to that reported for other higher plant species (Rabson et al. 1962, Lee and Whittingham 1974).

The paper chromatography systems devised for isolating glycolate, glycine, and serine from crude extracts of higher plant leaves should be of use to other researchers. Problems of glycolate volatility encountered in acidic solvents (Voskresenskaya et al. 1970, Pedersen et al. 1966b) and of glycolate-glycerate overlap (Bassham and Kirk 1973) were eliminated. The problem of glycine-serine-glucose overlap encountered with the widely used solvent system described by Pedersen et al. (1966b) was also eliminated. It was not necessary to resort to electrophoresis or column chromatography in order to achieve the desired separations.

E. Conclusions and Summary

A method for successfully carrying out kinetic tracer studies of steady state photosynthesis with labeled carbon dioxide by whole leaves

was developed. Advantages of the steady state apparatus and techniques developed include:

1) The capability for taking fifteen samples (of four leaflets each) provides enough points to define clearly the metabolic steady state.

2) Sufficient points are provided for to allow changes resulting from perturbation of steady state photosynthesis to be investigated. Samples can be removed from the exposure chamber before, during, and after the perturbation has occurred. A single set of leaflets, selected at one time, and simultaneously exposed to $^{14}CO_2$ can be used. This eliminates artifacts which can arise due to procedures which involve selection and exposure of leaflets at two different times to examine photosynthesis under two different conditions.

3) The capability for maintenance of labeled carbon dioxide concentration allows for experiments of long duration. The system has the further capability, through the use of the gas reservoirs and $^{14}CO_2$ supply device, of allowing for changes to be made in the $^{14}CO_2$ concentration during an experiment. The specific radioactivity of the $^{14}CO_2$ used is known.

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

5) The wells provided for leaflet petiole immersion during photosynthesis experiments allow for chemical treatment of the leaflets while they are exposed to labeled gas.

6) The use of whole leaflets may help to overcome disadvantages encountered with leaf discs, such as the effects of metabolism in injured cells.

New two-dimensional paper chromatography systems were developed for analysis of labeled glycolate, glycine and serine. The systems provide simple and economic means of separating those three materials from complex whole leaflet extracts.

Steady state photosynthesis by alfalfa leaflets with $^{14}CO_2$ was investigated and conclusions about carbon flow in those leaflets were drawn based upon labeling data. Many metabolite pools were observed to saturate with ^{14}C including the observed RPP cycle intermediates, TCA cycle intermediates, sucrose synthesis intermediates, and some amino acids. Steady state pool sizes of the identified RPP cycle intermediates were small relative to the total ^{14}C incorporation into soluble products. ^{14}C was rapidly used in the synthesis of other compounds. The major soluble labeled product was sucrose. Sucrose did not reach saturation due to the large size of that pool and the end product nature of sucrose production. Tracer carbon flow to the TCA cycle appeared to be occurring by the paths shown in Fig. 23. Labeling of materials usually associated with glycolate pathway metabolism--glycerate, glycine, serine--was substantial. However, based on the labeling curves, it was concluded that glycerate was synthesized directly from 3-PGA and not from glycolate. That glycerate synthesis reaction is the first step in a metabolic pathway which can substitute for the less efficient glycolate pathway.

In the following chapter the techniques and apparatus developed will be used to investigate perturbed steady state photosynthesis in whole alfalfa leaflets.

CHAPTER II

PHOTOSYNTHESIS BY ALFALFA LEAFLETS: HALF SATURATING AND SATURATING CARBON DIOXIDE CONCENTRATIONS

A. Introduction

In recent years there has been an expansion of interest in the growth of plants in atmospheres of elevated carbon dioxide level which cause increased rates of production (Zelitch 1971, Wittwer 1974, Wittwer and Robb 1964, Ford and Thorne 1967, Bishop and Whittingham 1968). This interest is related to investigations which showed that elevated levels of carbon dioxide increase photosynthetic rates (Gaastra 1959). The use of increased levels of carbon dioxide, perhaps made practical through utilization of CO_2 wells and plastic field coverings (Wittwer 1974), may be particularly of benefit in the agricultural production of legumes such as alfalfa. Elevated levels of CO_2 appear to increase nitrogen fixation by legumes to a substantial extent (Hardy and Havelka 1975). Alfalfa is an extremely good producer of high quality protein (Stahman 1968) and can be a source of a substantial amount of protein potentially suitable for direct human consumption (Edwards et al. 1975). Given the possible advantages to mankind of growing alfalfa at elevated CO_2 levels, I decided to investigate the effect of CO_2 concentration on alfalfa photosynthetic metabolism using ^{14}C as a tracer. My general goal was to uncover the biochemical effects of elevated CO_2 concentration on alfalfa photosynthetic metabolism, and also to learn more about its basic physiological gas exchange parameters.

Alfalfa appears to fall into the class of plants known as C_3 plants (Brown et al. 1972). That class includes most crop species.

For example, it includes rice, barley, oats, wheat, soybeans (Zelitch 1975a) and is distinct from the C_4 class of plants. Maize, sorghum, and sugarcane are the only known major C_4 crop plants (Chollet and Ogren 1975). The two classes of plants differ in numerous ways. Several of the apparent differences are:

1) C_3 plants utilize the enzyme ribulose-1,5-diphosphate carboxylase to catalyze the major part of their initial fixation of CO_2. The initially fixed carbon dioxide first appears in 3-PGA (Black 1973). C_4 plants make use of phosphoenolpyruvate carboxylase to initially bind carbon dioxide, the newly fixed carbon initially appears in oxaloacetate, CO_2 is later released and refixed internally by ribulose-1,5-diphosphate carboxylase (Black 1973).

2) Laetsch (1974) has emphasized the anatomical distinction noted between C_3 and C_4 plants. With one possible exception (recently described by Shomer-Ilan et al. 1975) all C_4 plants have a radial arrangement of chlorenchyma cells around leaf vascular bundles. The chlorenchyma is itself differentiated into two types of cells. An inner layer consisting of large thick walled cells containing large chloroplasts (the bundle sheath) and one or more outer layers of palisade-like mesophyll cells containing chloroplasts (Laetsch 1974, Chollet and Ogren 1975). This arrangement has been referred to as the Kranz anatomy. In the possible exception referred to above (Shomer-Ilan et al. 1975), a plant considered by the authors to be in the C_4 class has both types of chlorenchymatous cells but they are not radially arranged around the vascular bundle. One popular model of C_4 photosynthesis holds that the carbon dioxide is first fixed into OAA by the mesophyll cells, and is eventually transported into the bundle sheath cells where the CO_2 is released and finally refixed by

ribulose diphosphate carboxylase (Black 1973, Chollet and Ogren 1975). The 3-PGA then formed can go on to use in general plant metabolism. An alternative model has been discussed by Laetsch (1974).

3) C_3 plants have CO_2 compensation points (air level oxygen, approximately 25 C) in the range of 30-70 ppm while C_4 plants have CO_2 compensation points of from 0-10 ppm (Black 1973, Moss 1962, Krenzer et al. 1975). The CO_2 compensation point (or concentration) is defined operationally as the equilibrium level a plant attains when it is allowed to lower the CO_2 concentration in a closed system. At that level of CO_2 the rate of photosynthetic CO_2 uptake equals the total rates of respiratory release. Numerous environmental factors can affect the compensation point found for a species (Zelitch 1975b). The CO_2 compensation points of C_3 species are affected by both temperature (Joliffe and Tregunna 1968) and oxygen content of the surrounding atmosphere (Forrester et al. 1966a).

4) Inhibition of the net photosynthetic rate is observed in C_3 plants (high illumination, 300 ppm CO_2) when the oxygen level in the surrounding atmosphere is increased from 2 to 21%, but C_4 photosynthesis is essentially unaffected by such a change (Black 1973, Zelitch 1971).

5) C_3 plants generally seem to have lower crop growth and net photosynthetic rates than C_4 plants at air level oxygen and carbon dioxide and at temperatures near 25 C (Zelitch 1971 and 1973, Black 1973). However, some C_3 plants have very high rates of net CO_2 fixation (McNaughton and Fullem 1970) and under conditions of low temperature, C_3 plants can have higher rates of net assimilation than C_4 plants (Bjorkman and Berry 1973).

6) An oxygen stimulated post-illumination CO_2 outburst occurs in C_3 plants but not in C_4 plants (Decker 1955, Zelitch 1971, Jackson

and Volk 1970, Forrester 1966b).

7) Extrapolation of the curve relating apparent (net) photosynthesis and CO_2 concentration in C_3 plants (air level oxygen, 25 C) to zero CO_2 results in a large negative value of fixation that is greater than measured dark respiration (Decker 1957, Jackson and Volk 1970). In corn, a C_4 plant, the extrapolated value was zero (Hew et al. 1969).

Alfalfa leaves capable of photosynthesis have been reported by Huffaker et al. (1970) to have significantly greater (approximately 15 fold) ribulose diphosphate carboxylase activity than phosphoenolpyruvate carboxylase activity. The phosphoenolpyruvate carboxylase activity reported was of the order found in several C_3 plants and much less than that found in C_4 plants by Ting and Osmond (1973a) using similar assay conditions. Hatch (1971) has reported C_4 plants to have significantly greater phosphoenolpyruvate carboxylase activity than ribulose diphosphate carboxylase activity. Other enzymatic activity data supporting the C_3 classification of alfalfa is that the ratio of the 3-P-glycerate phosphatase and P-glycolate phosphatase activities in alfalfa was 1/8.8 in general agreement with the ratio found in C_3 plants and lower than that generally found in C_4 plants (Randall et al. 1971). Classification of alfalfa as a C_3 plant is also supported by available gas exchange measurements. Krenzer et al. (1975) reported alfalfa to be a high CO_2 compensation point species. Foutz et al. (1976) observed a sizeable post-illumination CO_2 outburst in alfalfa.

The gas exchange differences between C_3 and C_4 plants mentioned earlier (differences in compensation points, oxygen sensitivity of the

net photosynthetic rate, crop growth and photosynthetic rates, CO_2 outburst, extrapolated fixation value) have generally been attributed to the existence of a special type of measurable respiration (photorespiration) occurring simultaneously with photosynthesis in C_3 plants but not C_4 plants (Chollet and Ogren 1975, Zelitch 1975a and 1971, Jackson and Volk 1970). Also mentioned by some authors, as a contributing factor, is a possible direct inhibition of photosynthesis by O_2 which is believed to be closely associated with photorespiration (Laing et al. 1974, Chollet and Ogren 1975, Forrester et al. 1966a). Light is required for the synthesis of the photorespiratory substrate but not for its subsequent oxidation to CO_2.

There is substantial evidence that glycolate is the photorespiratory substrate (Jackson and Volk 1970, Tolbert 1973a, Zelitch 1975a, Chollet and Ogren 1975). Both photorespiration (as measured by gas exchange) and glycolate metabolism (its synthesis and further oxidation) appear to be stimulated by O_2 and repressed by very high levels of CO_2. Photosynthesis and plant productivity on the contrary are stimulated by CO_2 and inhibited by oxygen (Chollet and Ogren 1975, Zelitch 1975a and 1971, Jackson and Volk 1970, Gaastra 1959, Tolbert 1973a). There is disagreement in the literature regarding the mechanism of glycolate formation and the predominant mechanism of photorespiratory CO_2 release. Zelitch (1965) has presented evidence for glycolate synthesis in the light by reactions that are largely separate from those of the RPP cycle. Wilson and Calvin (1955) suggested that glycolate arises from oxidation of the glycolyl moiety transferred in the transketolase reactions of the RPP cycle. Other authors suggest it arises from the RuDP carboxylase mediated oxidation of RuDP by O_2 to produce P-glycolate which is then

hydrolyzed to glycolate (Bowes et al. 1971, Chollet and Ogren 1975, see also the earlier work of Bassham and Kirk 1962). Several contributing mechanisms are of course possible. After its synthesis, glycolate may be oxidized at least in part to photorespiratory CO_2 by two possible pathways. Both involve initial oxidation of glycolate to glyoxylate. One proposed path of CO_2 release is the sequence glycolate→glyoxylate→glycine→serine + CO_2 which is part of what is commonly called the glycolate pathway (Tolbert 1973a and 1971). Another proposed path involves the direct oxidative decarboxylation of glyoxylate (Zelitch 1975a). There is disagreement as to which path predominates (Zelitch 1975a and b, Chollet and Ogren 1975).

The absence of photorespiratory CO_2 release in C_4 plants has been attributed to mechanisms which have their origin in the anatomy and primary carboxylation reaction of C_4 plants. One suggested mechanism is that C_4 plants may be able to refix internally (using PEP carboxylase) any CO_2 released in the bundle sheath cells by photorespiratory mechanisms similar to those in C_3 plant cells. Refixation would occur in the mesophyll cells before the CO_2 escaped from the leaf. Another suggested mechanism is that PEP carboxylase in the mesophyll cells of C_4 plants concentrates CO_2 in the bundle sheath cells. The high level of CO_2 brought about at the site of the RuDP carboxylase reaction then limits glycolate formation, prevents the occurrence of measurable photorespiration, and reduces the inhibition of photosynthesis by O_2 (Chollet and Ogren 1975). Conditions of higher CO_2 than is present in air bring about increased photosynthetic rates and productivity as was mentioned earlier. In C_3 plants these increases may be partially attributable to higher environmental CO_2 level achieving the same physiological end

as C_4 plant anatomy and biochemistry: both the repression of photorespiration (Zelitch 1971, Zelitch 1975a) and of the associated O_2 inhibition of photosynthesis itself (Chollet and Ogren 1975).

Previous investigation of the effects of $^{14}CO_2$ concentration on higher plant photosynthetic metabolism studied plant species other than alfalfa. Most did not examine changes in labeling occurring as a function of time during perturbed steady state photosynthesis with $^{14}CO_2$, a technique reviewed by Bassham (1973). For example Bishop and Whittingham (1968), Osmond and Bjorkman (1972), Snyder and Tolbert (1974) and Lee and Whittingham (1974) conducted nonkinetic experiments in the sense that samples were not removed sequentially as a function of $^{14}CO_2$ exposure time. Leaf material was exposed to fixed $^{14}CO_2$ concentrations for fixed periods of time. Mahon et al. (1974) did remove samples as a function of time; however maximum $^{14}CO_2$ exposure was for an insufficient period of time to fully label even PGA. Some investigators concerned themselves primarily with metabolites of the glycolate pathway (Snyder and Tolbert 1974, Mahon et al. 1974) rather than with the flow of carbon to all early metabolites under conditions of differing CO_2 concentration. One study of bean leaf disc metabolism (Tamas and Bidwell 1970) examined carbon labeling changes occurring when approximately air level $^{14}CO_2$ concentration was altered to virtually zero (0.002%) $^{14}CO_2$ concentration. In an approach complementary to those investigating $^{14}CO_2$ metabolism, Madsen (1974) examined total pool sizes of several metabolites in tomato plants grown with different levels of $^{12}CO_2$ rather than looking at $^{14}CO_2$ incorporation into active photosynthetic pools.

As described in Chapter I, I recently developed an apparatus and

a set of experimental techniques that allows me to obtain and follow steady state photosynthesis with $^{14}CO_2$ by whole alfalfa leaflets. With it I can carry out kinetic tracer analysis of the flow of photosynthate during perturbed steady state photosynthesis by whole leaflets to a degree that was not previously possible. In this chapter I present information obtained in such an investigation of carbon labeling changes occurring in alfalfa leaflets when the $^{14}CO_2$ level to which they are exposed is varied between half saturation and saturation. General tracer carbon flow changes occurring in alfalfa transferred from half saturating to saturating $^{14}CO_2$ conditions are discussed as regards labeling of carbohydrates, RPP cycle intermediates, tricarboxylic acid cycle intermediates, and amino acids. Snyder and Tolbert (1974) recently presented data in apparent disagreement with the idea that high levels of CO_2 suppress glycolate pathway metabolite production and photorespiration. They indicated that their results suggest that increasing CO_2 concentration does not necessarily decrease the absolute amount of glycine and serine synthesis, and therefore does not decrease photorespiration (equated by them with carbon flow through the glycolate pathway). I, however, present data indicating that in alfalfa glycolate and glycine production is inhibited by saturating levels of CO_2, while serine production is not. The apparently different origins of serine and glycine in alfalfa are discussed. Evidence is presented that serine is produced predominantly from PGA and not from glycolate under my conditions. The possibility of such a path existing was mentioned by Randall et al. (1971) as an alternate metabolic pathway to glycolate metabolism and photorespiration. I had previously concluded, based on nonperturbed steady state data (Chapter I), that glycerate is produced

in alfalfa by that alternate path. I also present data on the compensation point of alfalfa, and the variation of alfalfa's photosynthetic rate with CO_2 and oxygen concentration.

B. Materials and Methods

1. 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 growth chamber at 3000 ft-c with a 9 hr light period and a 15 hr dark period both at 15 C. Plants were fertilized with modified Hoagland's solution twice weekly. The light source used was fluorescent lamps supplemented by incandescent lamps. For experimental purposes leaflets were excised with their petioles under water after 1-2 hr of light. Only the central leaflets (generally 45-65 mg) from the second through fourth unfolded leaves were used.

2. Steady State Gas Circulation System. The closed gas circulation system used was as described earlier (Chapter I, Fig. 1). It provides for gas circulation by means of a diaphragm pump and continuous monitoring and recording of the levels of $^{14}CO_2$, $^{12}CO_2$ and oxygen in the circulating gas. In line removable gas reservoirs and a removable Ascarite tube provide for rapid adjustment of the carbon dioxide level in the system. Bubblers (inline) provide for maintenance of the humidity level in the circulating gas stream. Gas concentration detectors are standardized against gases of known composition (and room air in the case of the O_2 detector).

3. $^{14}CO_2$ and $^{12}CO_2$ Supply Systems. The carbon dioxide supply systems were described in detail (Chapter I, Figs. 3a and b). Using them, gas can be manually released into the circulating gas stream to

maintain or change the CO_2 level in that stream. Regulation of gas release is accomplished using a Millaflo micrometering regulator. Both supply systems use 21% CO_2 (total) in nitrogen. The specific radioactivity of the $^{14}CO_2$ in the labeled gas supply during the experiments reported here was 22.3 μCi/μmole in one case, and 25.8 μCi/μmole in the other.

4. Steady State Leaf Exposure System. The leaf exposure system (the leaf exposure device (LED) and its associated light banks) was described earlier (Chapter I, Fig. 4). It provides for the exposure of up to 60 alfalfa leaflets, removable independently in groups of four without disturbing the remaining leaflets. It provides for leaflet temperature control by means of water jackets and a water bath. Temperature within the leaf exposure chamber is determined by means of a thermocouple. Leaflets are exposed to the circulating gas stream from both above and below. Leaflet petioles are immersed in water throughout photosynthetic experiments. That procedure, and the use of inline bubblers, prevents leaflet dehydration. Lighting is provided by two banks of fluorescent lamps. Light intensity at the upper and lower surfaces of the leaflets is 1800 ft-c (approximately equivalent to a total illumination intensity of 3600 ft-c from a single light bank above the leaflets).

5. Photosynthetic $^{12}CO_2$ Fixation as a Function of $^{12}CO_2$ Concentration. Sixty leaflets were exposed to unlabeled carbon dioxide in air at various concentrations of CO_2 using the gas circulation and leaf exposure systems described above. The carbon dioxide concentrations were changed by manipulation of the gas reservoirs (Fig. 1, two additional reservoirs of approximately 0.58 liter and 5 liters were added), by use of the $^{12}CO_2$ supply system, and through use of Ascarite.

The oxygen concentration throughout the experiment was 22.7% ± 0.1%. The inline bubbler (Fig. 1) contained 10 ml of pH 4.7 citrate buffer (0.05 M citrate). Flow rates were 1 LPM through the $^{12}CO_2$ analyzer, 0.2 LPM through the O_2 analyzer, and 4 LPM through the LED. The overall gas volume of the system was approximately 8 liters, although a 5 liter gas reservoir was removed from the circulation system for determination of the carbon dioxide compensation point. The bubbler temperature was 22 C and the LED water jackets and water bath were maintained at 15 C. The temperature measured within the leaf exposure chamber was 18 C. Net photosynthetic rates were calculated from the slope of the $^{12}CO_2$ recorder trace (CO_2 concentration as a function of time), known system gas volume, and the total leaflet Chl content (see below). Unless otherwise indicated, all photosynthetic rates given in this thesis are net or apparent rates of photosynthesis. The photosynthetic rate at each carbon dioxide concentration was determined after preliminary photosynthesis at that level for approximately 10 min. The CO_2 level was maintained during those preliminary periods by means of the $^{12}CO_2$ gas supply device. Carbon dioxide flow from the supply device was then stopped and the rate of uptake followed for periods of two minutes. Rates are reported at the average CO_2 concentration occurring during the 2 min periods. The concentration decrease during the measurement period was of the order of 5-20% of its initial value. The photosynthetic rate was first determined at approximately air level CO_2, and that measurement was repeated after the rate measurements at elevated CO_2 concentration. Those values agreed within experimental error. The CO_2 compensation point under these conditions was determined from above (by allowing photosynthesis to occur until the CO_2 level was reduced to its compensation point value) and from below (allowing

respiration to occur in the light from approximately 5 ppm CO_2 until the compensation point was attained). Instrument standardization was checked at the conclusion of the experiment. Following the photosynthetic experiment, the leaflets were ground as described earlier (Chapter I) and extracted with magnetic stirring in 80% ethyl alcohol for 1-1/2 hr at 20 C in the dark. Chl content was then determined using the method of Bruinsma (1963). Spectra were corrected for light scattering, and the Chl content was taken to be the average of the values obtained from Bruinsma's (1963) two equations. Photosynthesis rates are reported in units of μmoles CO_2/hr·mg Chl.

6. Photosynthetic $^{12}CO_2$ Fixation and Dark Respiration as a Function of Oxygen Concentration. As a preliminary experiment, the effect of O_2 concentration on the determinations of $^{12}CO_2$ concentration obtained using the Beckman 315B $^{12}CO_2$ analyzer was examined. Gale et al. (1975), using a Grubb Parsons Differential Infrared Gas Analyzer (Model SB2), reported a dependence of the instrument CO_2 calibration factor on oxygen concentration, particularly above 30% oxygen. To investigate whether this effect was present in our system, a fixed volume (19 ml) of 1.088% $^{12}CO_2$ in air was released into a circulating gas stream (approximate volume 700 ml) containing various proportions of nitrogen and oxygen but no carbon dioxide. The circulating gas stream was then allowed to mix thoroughly. The meter reading of the Beckman 315B after thorough mixing was independent of the equilibrium oxygen concentration. The final CO_2 concentration in the circulating stream was 0.031%. Final O_2 concentrations after mixing were approximately 1.0%, 20%, 58%, and 92%. It was concluded that the Beckman 315B $^{12}CO_2$ readings do not require correction for stream oxygen content.

Forty-five leaflets were exposed to unlabeled carbon dioxide in various mixtures of nitrogen and oxygen. The carbon dioxide concentration was generally maintained in the range of 0.032% CO_2 to 0.037% CO_2 through use of the $^{12}CO_2$ gas supply device. The oxygen-nitrogen proportions were changed through manipulation of the gas reservoirs and through slow addition of 100% O_2 or N_2 as necessary. The leaflets were exposed to the various gas mixtures using the LED and gas circulation system described above. The inline bubbler contained 10 ml of pH 4.3 citrate buffer (0.05 M citrate). Flow rates through the LED, O_2 and $^{12}CO_2$ analyzers were as given in the preceding section. The bubbler temperature was 25 C while the LED water bath and water jackets were maintained at 20 C. The temperature measured in the leaf chamber was 23 C. Photosynthetic rates were determined from the slope of the $^{12}CO_2$ recorder trace, known system gas volume and leaflet Chl content. The rate at each oxygen level was determined by measuring the time for the CO_2 concentration to fall from approximately 0.037% CO_2 to 0.032% following a preliminary 10 min period of photosynthesis at that oxygen level. The flow from the $^{12}CO_2$ gas supply device was stopped during the rate determinations. Rates are reported at an average CO_2 concentration of 0.0345%. The photosynthetic rate was first determined at approximately 18% O_2 and the final determination was made at approximately 21% O_2. The photosynthetic rate was generally determined twice in succession at each oxygen level. Following the final rate determination the leaflets were allowed to reduce the $^{12}CO_2$ level to their compensation point under these conditions (23 C, 21% O_2). Instrument standardization was checked at the conclusion of the experiment. Leaflets were ground and their Chl content determined as described in

the previous section. Photosynthetic rates are reported in units of μmoles CO_2/hr·mg Chl.

In a second experiment, the effect of O_2 on alfalfa leaf photosynthesis, as well as its effect on dark respiration, was examined at 17 C ± 1 C (leaf exposure chamber temperature). Fifty alfalfa leaflets containing 3.28 mg Chl were used. Measurements were made essentially as described above. Photosynthetic rate values were determined at 20% and 2% oxygen, 0.025% CO_2. Following those rate determinations the lights were turned off and dark respiration was measured at 20% and 2% oxygen, 0.03% CO_2.

7. Perturbed Steady State $^{14}CO_2$ Fixation. Two similar experiments were conducted in which alfalfa leaflets were exposed to $^{14}CO_2$ in air. In the first, experiment A, leaflets were exposed to $^{12}CO_2$ for a period of time previously shown to be sufficient to bring about steady state photosynthesis (Chapter I). That period was followed by exposure to half saturating $^{14}CO_2$. After a period of fixation of tracer gas, the $^{14}CO_2$ concentration was raised to greater than the CO_2 saturation value. In the second experiment, experiment B, the period of photosynthesis at greater than $^{14}CO_2$ saturation was followed by another period of fixation at approximately half saturating $^{14}CO_2$. The details of experiments A and B are given below and in Table 4. The specific radioactivity of the $^{14}CO_2$ was 22.3 μCi/μmole during experiment A, and 25.8 μCi/μmole during experiment B. As in Chapter I, $^{14}CO_2$ concentrations given refer to the total amount of carbon dioxide present during the tracer portions of the experiments. That is a concentration of "P% $^{14}CO_2$" means P% total carbon dioxide was present and it had a specific radioactivity of 22.3 μCi/μmole during experiment A and 25.8

Table 4. Conditions of Photosynthesis with $^{14}CO_2$

Experiment A	First Period		Second Period
$^{14}CO_2$ concentration (%)	0.024 ± 0.003		0.22 ± 0.03
Time (min)*	0 - 33		33 - 71
Light level (ft-c)**		3600	
Oxygen concentration (%)**		19.5 ± 1.5	
Temperature (C)**		18 ± 1	
$^{14}CO_2$ specific radioactivity** (μCi/μmole)		22.3	

Experiment B	First Period		Second Period	Third Period
$^{14}CO_2$ concentration (%)	0.026 ± 0.003		0.20 ± 0.03	0.026 ± 0.003
Time (min)*	0 - 29		29 - 61	61 - 98
Light level (ft-c)**		3600		
Oxygen concentration (%)**		21.2 ± 0.2		
Temperature (C)**		16 ± 1		
$^{14}CO_2$ specific radioactivity** (μCi/μmole)		25.8		

* Time was measured from the initiation of photosynthesis with $^{14}CO_2$.

** Unchanged throughout a given experiment.

μCi/μmole during experiment B.

Experiment A. Sixty alfalfa leaflets were inserted in the LED in the dark; the light banks were on but covered by opaque panels. The leaflets were then exposed to unlabeled $^{12}CO_2$ in air, and the panels blocking the light banks were withdrawn to initiate photosynthesis. The initial level of $^{12}CO_2$ was 0.059%. That level was allowed to decline to approximately 0.03% $^{12}CO_2$ as photosynthesis proceeded for 7 min. For an additional 8 min the level of $^{12}CO_2$ was maintained in the range 0.026 $\pm$ .003% using the $^{12}CO_2$ gas supply device. Then without altering any other variable, and by the use of the gas reservoirs of the steady state gas circulation system, the leaflets were exposed to $^{14}CO_2$. The circulating stream was then maintained at 0.024% $\pm$ 0.003% $^{14}CO_2$ for a period of 33 min using the $^{14}CO_2$ gas supply device. At that time, using the gas reservoirs of the circulation system, the $^{14}CO_2$ level supplied to the leaflets was increased to 0.22% $\pm$ .03% and maintained at that level for 38 min using the $^{14}CO_2$ gas supply device. Gas flow was 4 LPM. The LED water bath and water jacket temperature was 15 C $\pm$ 1 C. The leaf exposure chamber temperature was 18 C $\pm$ 1 C. The oxygen concentration throughout the experiment was 19.5% $\pm$ 1.5%. An inline bubbler was always present in the gas circulation line (21 C, 10 ml deionized water). Fifteen samples (each containing four leaflets) were removed as a function of time during the $^{14}CO_2$ portions of the experiment. Seven were removed during the exposure to low level $^{14}CO_2$ (0.024%) and eight during the exposure to high level of $^{14}CO_2$ (0.22%).

Experiment B. Sixty alfalfa leaflets were inserted in the LED in the dark (opaque panels blocking the light banks), and the leaflets were exposed to 0.05% $^{12}CO_2$ in air. The panels blocking the light banks were then removed and photosynthesis began. The level of $^{12}CO_2$

was allowed to decline for approximately 7 min to 0.026% $^{12}CO_2$ so as to permit measurement of the rate of photosynthesis. That rate was measured using the $^{12}CO_2$ recorder plot at an average $^{12}CO_2$ concentration of 0.033%. The level of $^{12}CO_2$ was then held at 0.026% $\pm$ .003% for an additional period of 4 min using the $^{12}CO_2$ gas supply device. Then, as in experiment A, by use of the gas reservoirs, the leaflets were exposed to $^{14}CO_2$. The $^{14}CO_2$ level was maintained at 0.026% $\pm$ .003% for a period of 29 min using the $^{14}CO_2$ gas supply device. Following that period the $^{14}CO_2$ level in the gas stream was increased to 0.20% $\pm$ 0.03% by use of the gas reservoirs. It was maintained in that range for 32 min. Following the exposure to high concentration labeled CO_2 the $^{14}CO_2$ level in the circulating gas stream was returned to 0.026% $\pm$ 0.003% by use of the gas reservoirs. It was maintained in that range by the labeled gas supply device for 37 min. Gas flow was 4 LPM. The LED water bath and water jacket temperature was maintained at 13 C $\pm$ 1 C throughout the experiment. The leaf exposure chamber temperature was 16 C $\pm$ 1 C. The oxygen concentration was 21.2% $\pm$ 0.2%. An inline bubbler was always in the gas circulation line (7 ml pH 4.7 citrate buffer, 0.05 M citrate, 20 C). Fifteen samples (each containing 4 leaflets) were removed as a function of time during the $^{14}CO_2$ portions of the experiment. Four were removed during the initial low level (0.026%) period, six during the exposure to a high concentration of $^{14}CO_2$ (0.20%), and the last five during the final exposure to low level $^{14}CO_2$ (0.026%).

8. Treatment of Leaf Samples. Leaflets were frozen by, and stored in, liquid nitrogen immediately upon removal from the LED. Each sample (consisting of 4 leaflets) was ground as described in Chapter I. Leaflets were then extracted in approximately 13 ml of 80% ethyl alcohol

for 3.0 hr, 2 C, in the dark (with magnetic stirring) in the case of experiment A, and for 1.5 hrs, 22 C, in the dark (with magnetic stirring) in the case of experiment B. (Chl extraction under these conditions is equivalent, see Chapter I.) Suspensions were then centrifuged (IEC centrifuge) for 5 min at 2800 rpm. The supernatant was decanted, and the pellets were washed with an additional 2 ml of 80% ethyl alcohol which was combined with the supernatant. The chlorophyll content of the extract was determined by visible spectroscopy using the method of Bruinsma (1963) as described in Chapter I. The pellets were then extracted with 4 ml of 20% ethyl alcohol for 1 hr at 25 C. These suspensions were centrifuged at 2800 rpm for 10 min (IEC centrifuge) and the supernatant decanted. The pellets were extracted with 2 ml of H_2O, 30 min, 80 C $\pm$ 10 C, centrifuged as above, and the supernatant decanted. A final extraction with an additional 1.5 ml of H_2O, 95 C, 30 min was carried out and following centrifugation as above, the supernatant was removed using a pipet. The 80% ethyl alcohol, 20% ethyl alcohol, and the two water extracts were combined and thoroughly mixed. The final total extract volume of each sample was approximately 20 ml. The pellet was rinsed with 80% ethyl alcohol onto filter paper in a Büchner funnel, washed with several drops of formic acid, and dried by suction. The solid material and the filter paper which it was on was further dried in a vacuum dessicator over KOH and silica gel. The dried material and its associated paper was combusted (Packard Automatic Combustion Apparatus) to give data on $^{14}CO_2$ fixation into insoluble material as described earlier in Chapter I. The sample extracts were analyzed for fixation into soluble materials by liquid scintillation. Aliquots of each extract were acidified with formic acid, dried and counted as described earlier (Chapter I).

Results for fixation into insoluble and soluble products were expressed on the basis of microgram atoms of ^{14}C/mg Chl. The microgram atoms of ^{14}C were calculated by dividing the radioactive content of the product in μCi by the specific radioactivity of the entering $^{14}CO_2$ used (22.3 μCi/μmole in experiment A, 25.8 μCi/μmole in experiment B). In this way, as in Chapter I, the μg atoms of ^{14}C in a product corresponds to a given number of μmoles of administered labeled carbon dioxide. Values determined in that way permit direct comparison between the overall rate of fixation and ^{14}C accumulation in a product (whether the product considered is a class of metabolites or an individual metabolite as is the case below).

9. $^{14}CO_2$ Fixation into Specific Soluble Metabolites. Aliquots of the sample extracts were analyzed by two-dimensional paper chromatography systems a, c, and d described in Chapter I. In all cases fixation results were expressed on the basis of microgram atoms ^{14}C (calculated as described in the preceding section) fixed per mg Chl into each metabolite. Fixation into most metabolites was determined using system a (Figs. 8, 9). Fixation into glycolate was determined using system c (Fig. 11). Glycine, serine, and glucose label contents were determined using system d (Fig. 12).

C. Results

1. Photosynthetic $^{12}CO_2$ Fixation as a Function of Carbon Dioxide and Oxygen Concentration. The dependence of the alfalfa leaflets net photosynthetic rate upon carbon dioxide concentration (22.7% O_2, 18 C, 3600 ft-c) is presented in Fig. 24. At low levels of carbon dioxide the rate of photosynthesis increased with increasing carbon dioxide.

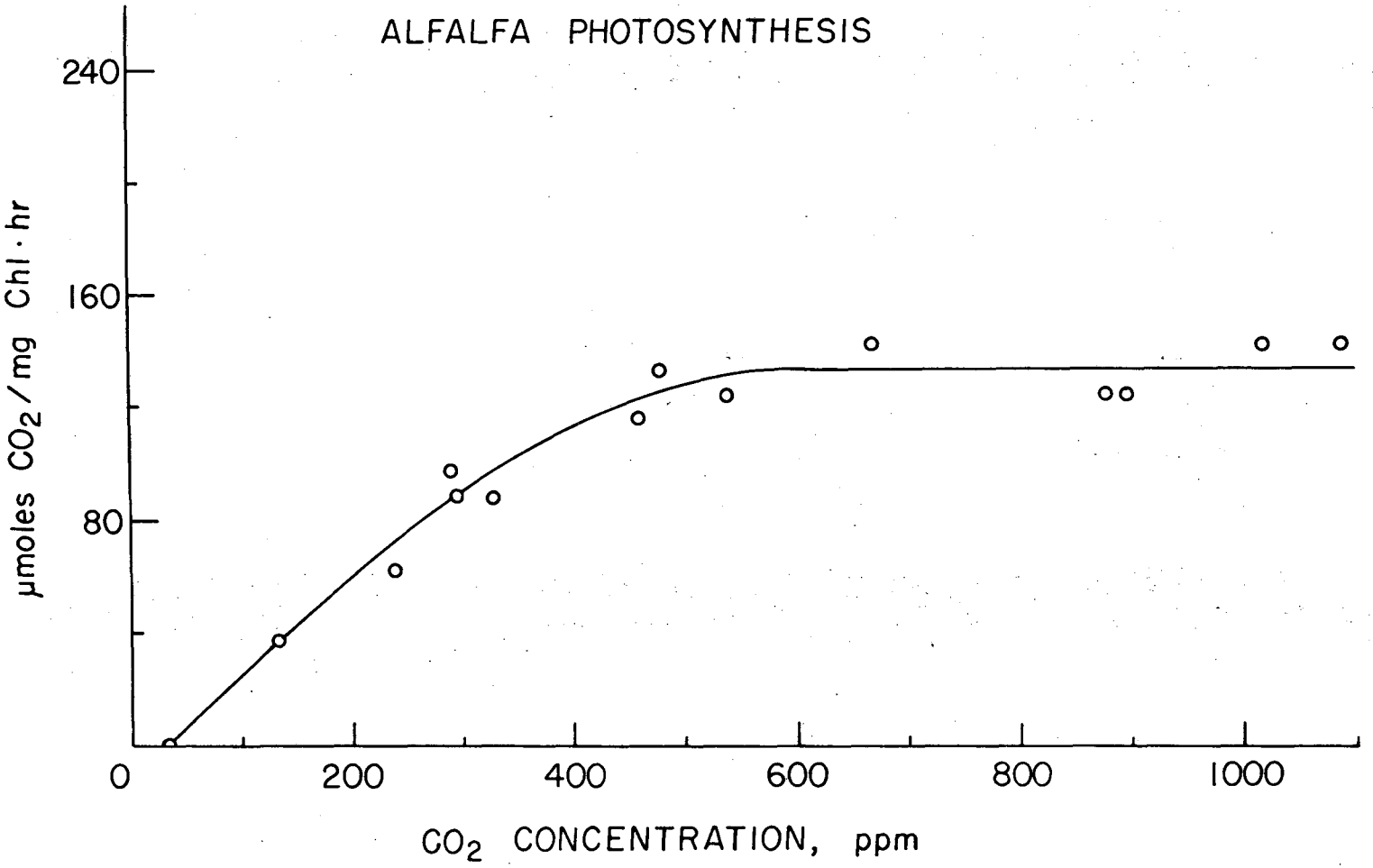

Figure 24. Photosynthesis in relation to carbon dioxide concentration. Alfalfa leaflets at 22.7% O_2, 18 C, and 3600 ft-c.

Photosynthesis became saturated with respect to CO_2 at approximately 0.055% CO_2. The photosynthetic rate at saturation was approximately 50% greater than that at air level CO_2 (0.03% CO_2), and approximately 70% greater than that at half saturation (0.0275% CO_2).

Extrapolation of the curve in Fig. 24 through the CO_2 compensation point to 0 ppm CO_2 gave a negative value of 16 μmoles CO_2/hr•mg Chl for net photosynthesis. Such extrapolated values have been used as rough estimates of the minimum measurable photorespiration occurring where photorespiration is thought of as the total CO_2 release occurring in the light (Canvin and Fock 1972, Zelitch 1971, Jackson and Volk 1970). The extrapolated value is equal to 21% of the net photosynthesis occurring at 0.025% CO_2. Dark respiration in the alfalfa leaflets utilized was estimated to be 9.4 μmoles CO_2/hr•mg Chl (see experimental determination reported below). Hence photorespiration estimated by extrapolation was 1.7 times dark respiration. Due to the disagreement as to the exact nature of photorespiration and general disagreement as to how it is to be best measured (Chollet and Ogren 1975, Jackson and Volk 1970, Ludlow and Jarvis 1971, Zelitch 1975b), all estimates of photorespiration are only crude indications of the extent to which that process is occurring. Such estimates of photorespiration are always in doubt as one is attempting to measure it while at least three complex processes are occurring simultaneously: photosynthesis, photorespiration, and perhaps mitochondrial respiration.

The rate of photosynthesis of the alfalfa leaflets was also found to be strongly dependent on the atmospheric oxygen content. Figure 25 presents the data obtained (0.0345% CO_2, 23 C, 3600 ft-c). The photosynthetic rate increased as the O_2 concentration was decreased. A photosynthetic rate increase of 50% was obtained upon lowering the

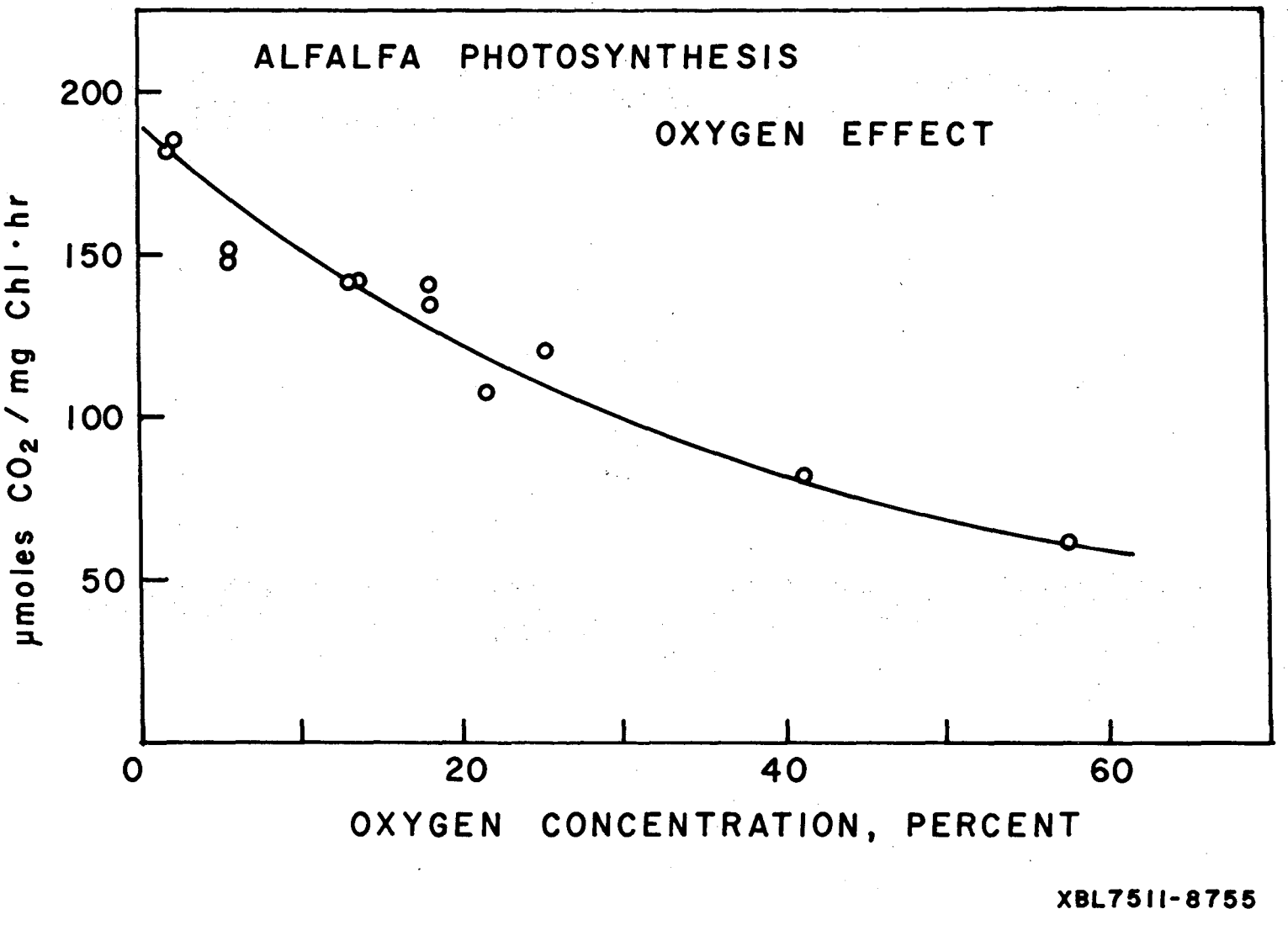

Figure 25. Photosynthesis in relation to oxygen concentration. Alfalfa leaflets at 0.0345% CO_2, 23 C, 3600 ft-c.

oxygen concentration from 21% to 2%. The rate of photosynthesis at 0.0345% CO_2 (22.7% O_2, 23 C, 3600 ft-c) was 115 μmoles CO_2/hr•mg Chl (Fig. 25). This value is in reasonable agreement with the data presented in Fig. 24 given the somewhat lower temperature at which that data was obtained. In the experiment conducted at 17 C, the net photosynthetic rate of the alfalfa leaflets at 0.025% CO_2 increased by 32% on lowering the O_2 concentration from 20% to 2%. Photosynthetic rates of 115 μmoles CO_2/hr•mg Chl (20% O_2) and 152 μmoles CO_2/hr•mg Chl (2% O_2) were observed. Only part of the O_2 inhibition of photosynthesis may be due to photorespiration (Ludwig and Canvin 1971).

2. Alfalfa Dark Respiration Rates and CO_2 Compensation Point. The dark respiration rates observed at 17 C were 9.4 μmoles CO_2/hr•mg Chl (20% O_2, 0.03% CO_2) and 7.3 μmoles CO_2/hr•mg Chl (2% O_2, 0.03% CO_2). The drop in the dark respiration rate observed on lowering the O_2 concentration was equal to only 1/16 of the photosynthetic rate increase occurring under similar conditions.

The alfalfa leaflets CO_2 compensation point was found to be 35 ppm at 18 C (22.7% O_2, 3600 ft-c) and 45 ppm at 23 C (21% O_2, 3600 ft-c).

3. Photosynthetic $^{14}CO_2$ Fixation: Half Saturating and Saturating $^{14}CO_2$ Concentrations.

a. General considerations and labeling of various classes of products. Selection of the $^{14}CO_2$ concentrations used was based on the data presented in Fig. 24. The products obtained on the paper chromatograms (Figs. 8, 9, 11, 12) were those discussed earlier in Chapter 1, and were generally similar to those reported in other higher plant species (Tamas and Bidwell 1970, Jensen and Bassham 1966).

The data obtained during experiments A and B for the total fixation of $^{14}CO_2$ and for the fixation of $^{14}CO_2$ into soluble products, is

presented in Figs. 26 and 27. Figures 28 and 29 present labeling data on the formation of insoluble products. The ^{14}C labeling rates calculated for ^{14}C fixation into total, soluble, and insoluble products using the data in Figs. 26-29 are presented in Table 5.

The photosynthetic rate determined using the $^{12}CO_2$ analyzer (Fig. 1) in experiment B, prior to the initiation of photosynthesis with $^{14}CO_2$, was 100 μmoles $^{12}CO_2$/hr•mg Chl at a carbon dioxide concentration of 0.033%. This is in good agreement with the total rate of $^{14}CO_2$ incorporation observed during the initial $^{14}CO_2$ period given the somewhat higher level of $^{12}CO_2$ (refer to Fig. 24).

The overall rate of photosynthesis approximately doubled in both experiments when the $^{14}CO_2$ concentration was increased from half saturating to saturating. This increase was reflected in both the rate of labeling of soluble and insoluble products. In both experiments, approximately 40% of the total rate increase was accounted for by an increased rate of labeling of soluble products, while approximately 60% is accounted for by an increased rate of labeling of insoluble products.

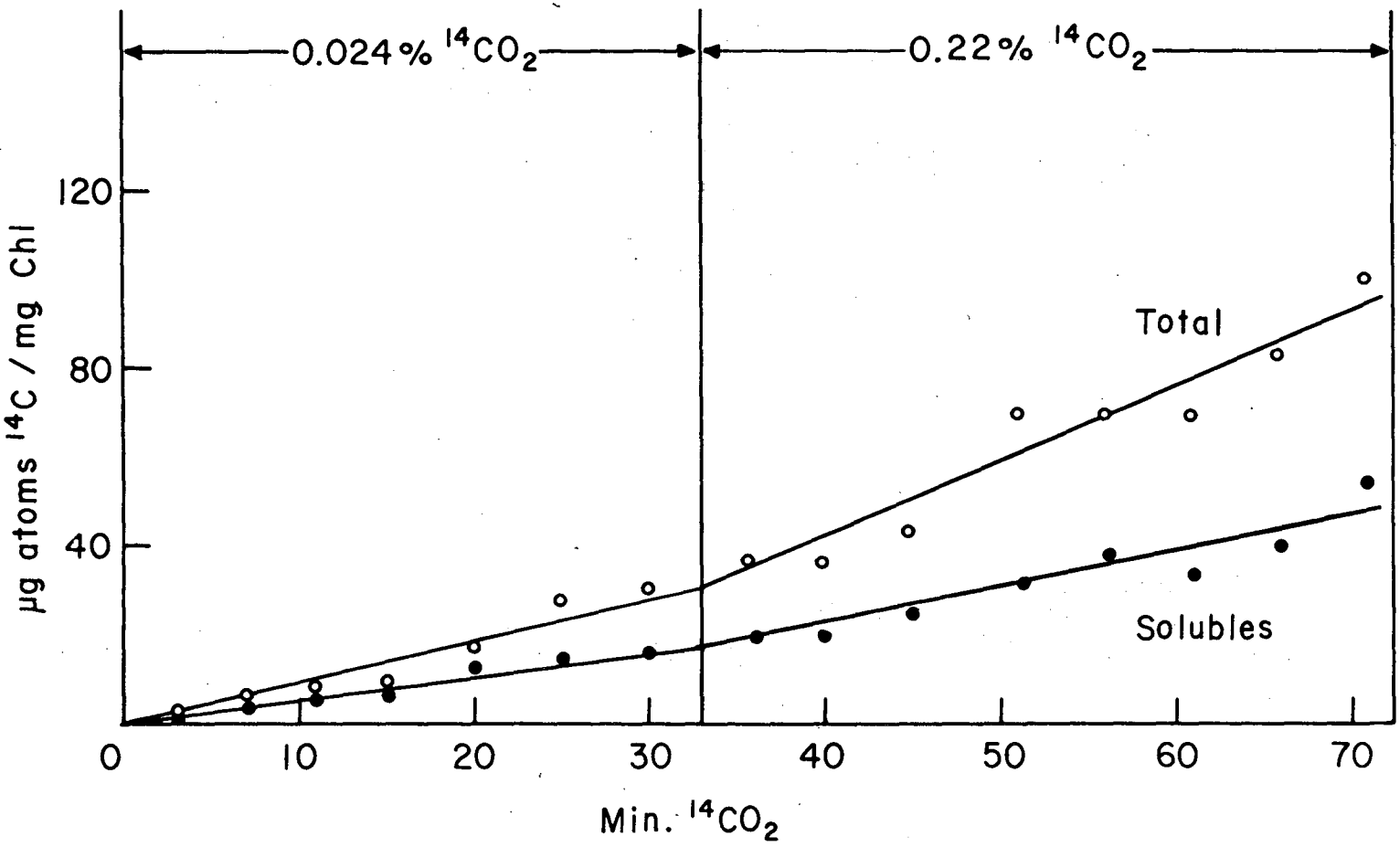

Figure 26. Total photosynthetic ^{14}C incorporation and incorporation of ^{14}C into soluble products by alfalfa leaflets during experiment A. The experimental conditions are given in Table 4.

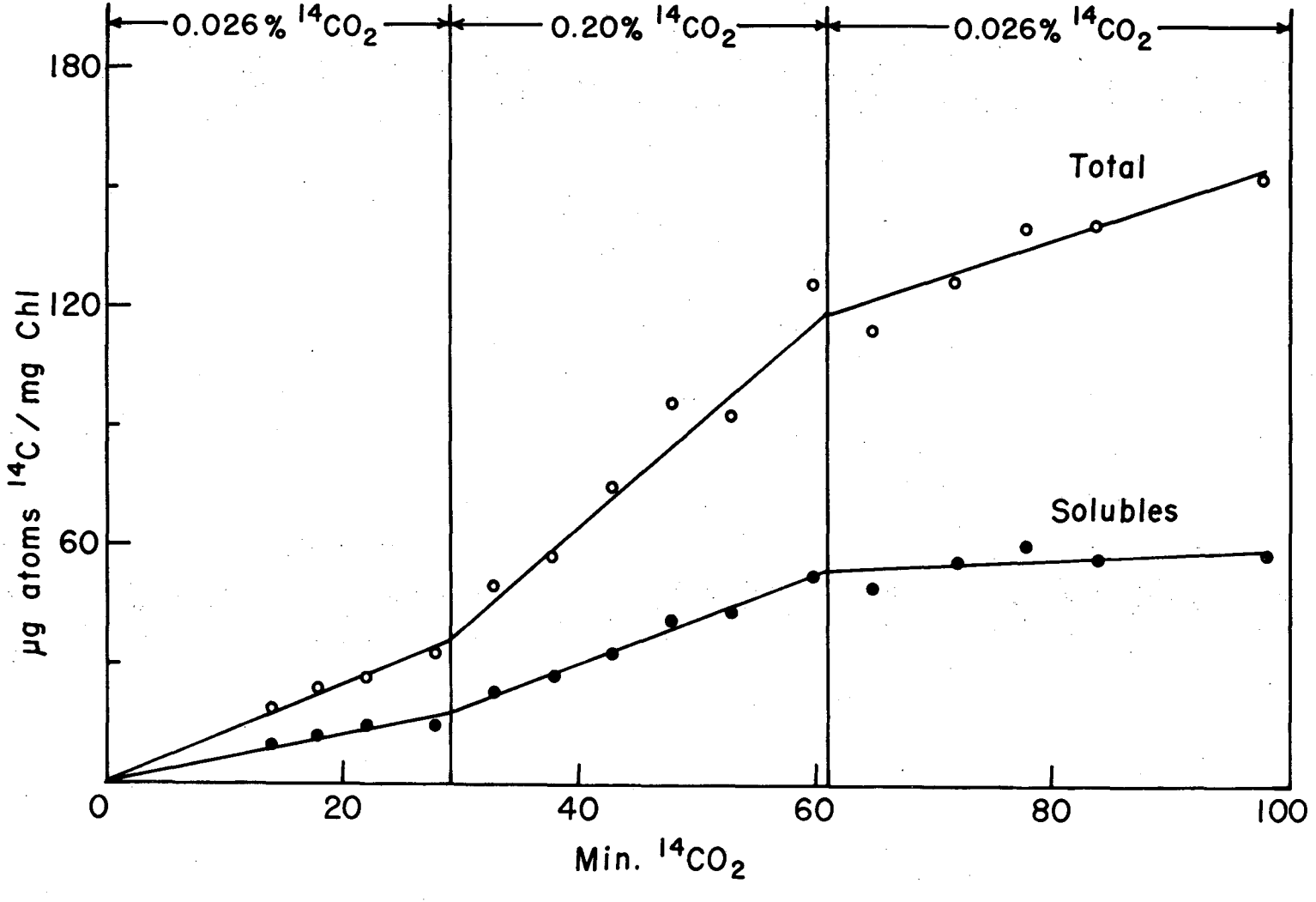

Figure 27. Total ^{14}C incorporation and incorporation into soluble products by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

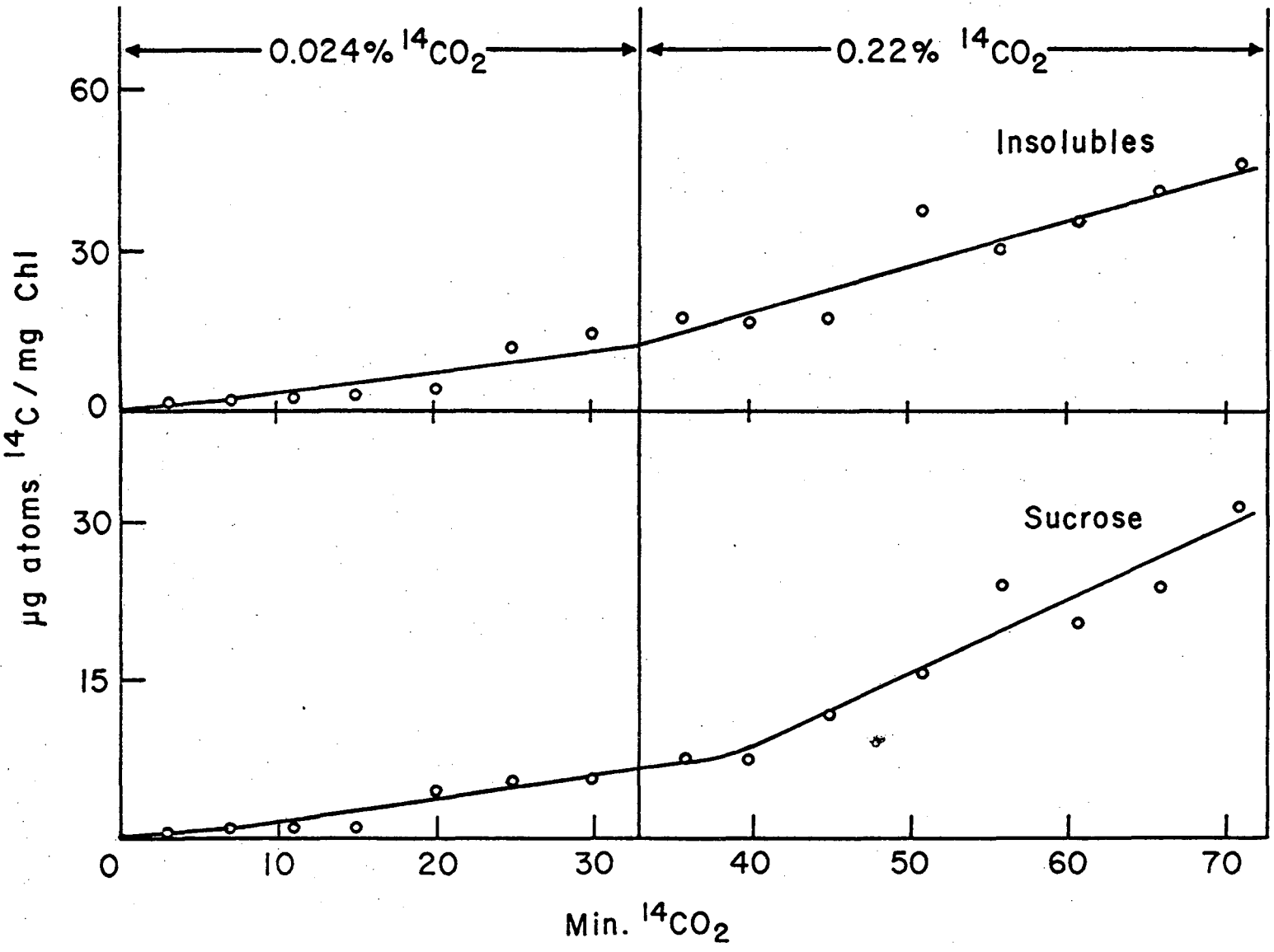

Figure 28. Incorporation of ^{14}C into insoluble products and into sucrose by alfalfa leaflets during experiment A. The experimental conditions are given in Table 4.

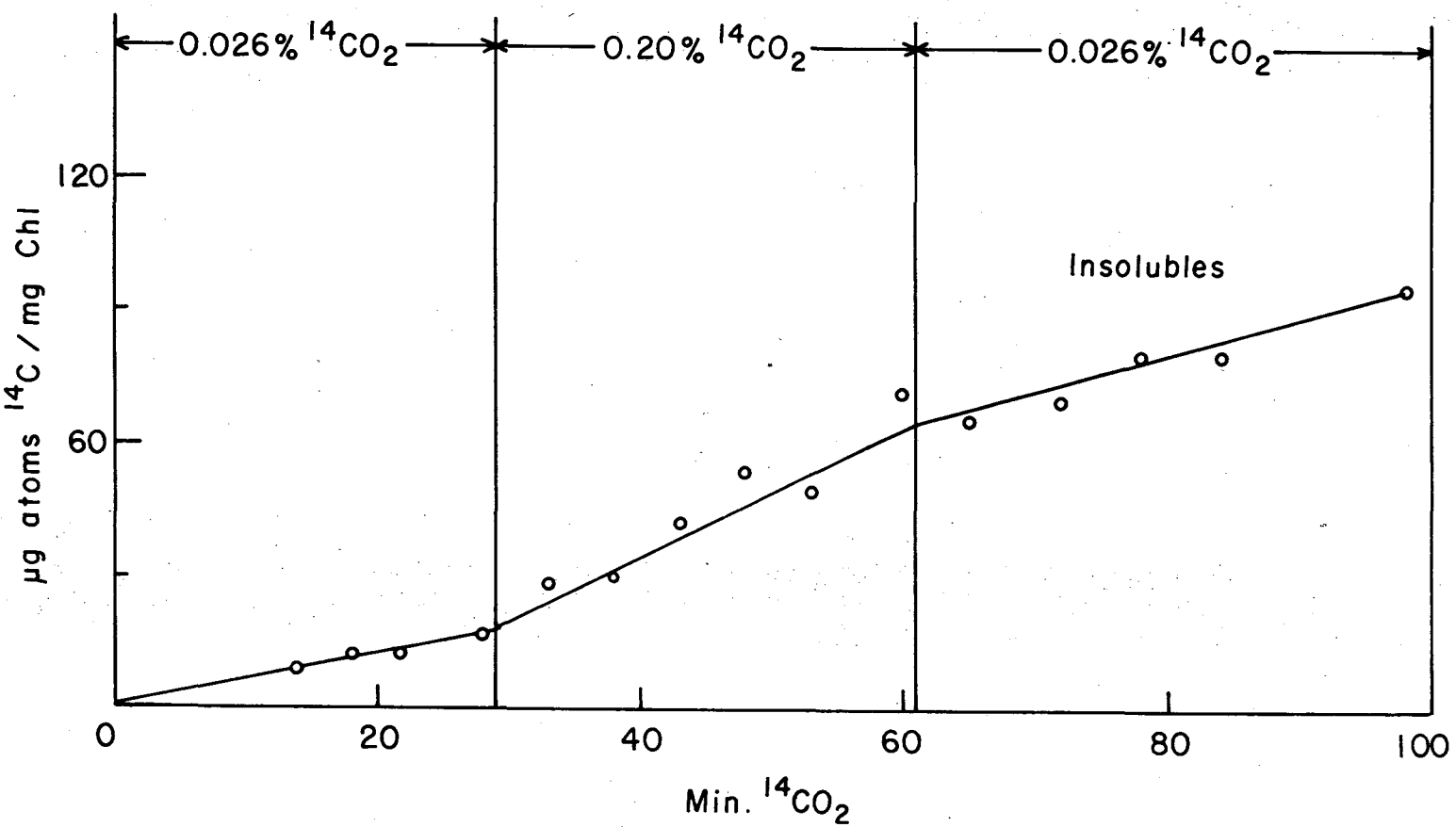

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Figure 29. Incorporation of ^{14}C into insoluble products by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

Table 5. ^{14}C Labeling Rates* of Total, Soluble, and Insoluble Products

Experiment A	First Period	Second Period
$^{14}CO_2$ concentration (%)	0.024	0.22
Labeling rates		
soluble products	32	52
insoluble products	23	53
total products	56	104

Experiment B	First Period	Second Period	Third Period
$^{14}CO_2$ concentration (%)	0.026	0.20	0.026
Labeling rates			
soluble products	36	64	12
insoluble products	38	84	52
total products	72	148	64

* All rates given in units of μg atoms ^{14}C/hr·mg Chl

In experiment B, during the final 0.026% $^{14}CO_2$ period (Table 5), the total photosynthetic rate returned to approximately the same value as was obtained during the first such period. The major part of the $^{14}CO_2$ incorporation rate in the final 0.026% $^{14}CO_2$ period was accounted for by additional labeling of insoluble materials (probably proteins, polysaccharides, etc.). Soluble pool label increased only slowly during that period.

b. Labeling curves of individual soluble metabolites. The labeling curves obtained were of the general types encountered

previously in Chapter I: When photosynthesis with $^{14}CO_2$ was initiated, some active metabolite pools eventually became saturated with ^{14}C. In such cases the saturation value level of label in the metabolite, its steady state level, is a direct measure of the amount of material in that active pool (neglecting possible contributions from endogenous carbon sources). In other cases photosynthesis with a given concentration of $^{14}CO_2$ did not continue for a sufficient time to saturate an active metabolite pool with ^{14}C. In those cases the amount of ^{14}C found in the metabolite is not a direct measure of its active pool size. It is instead a measure of the amount of that metabolite present which has arisen from recently fixed $^{14}CO_2$.

Labeling curves for PGA, and sugar mono and diphosphates from experiment A are presented in Figs. 30-32. Curves for those metabolites from experiment B are presented in Figs. 33-36. In experiment A, PGA (Fig. 30), the sugar diphosphates (Fig. 31) and sugar monophosphates (S7P + G6P, F6P; Figs. 31, 32) reached ^{14}C saturation early in the first period of photosynthesis with $^{14}CO_2$. Label saturation was reached after 10-20 min of photosynthesis with 0.024% $^{14}CO_2$. When the concentration of $^{14}CO_2$ was increased, the steady state levels of PGA and the sugar monophosphates S7P + G6P remained unchanged (Figs. 30, 31). The sugar diphosphates pool size increased somewhat towards the close of the experiment. The steady state level of F6P increased by 60% when the $^{14}CO_2$ concentration was raised to a saturating level (Fig. 32). It reached a new steady state level approximately twelve minutes after the $^{14}CO_2$ increase. The pattern of change was somewhat different in experiment B. In that experiment PGA (Fig. 33), the sugar diphosphates (Fig. 34), S7P + G6P (Fig. 35) and F6P (Fig. 36) all reached label saturation during the first 20 min of the initial period of photosynthesis

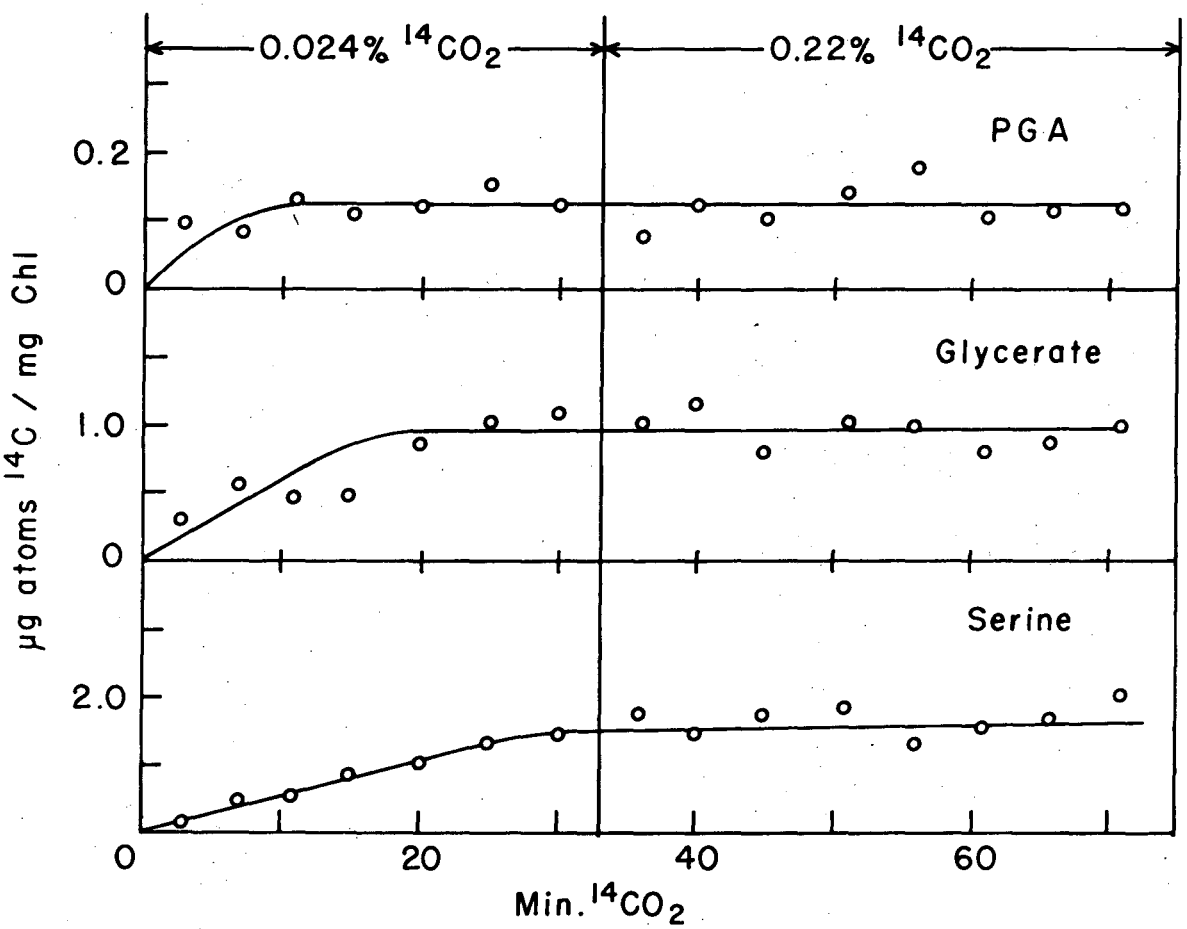
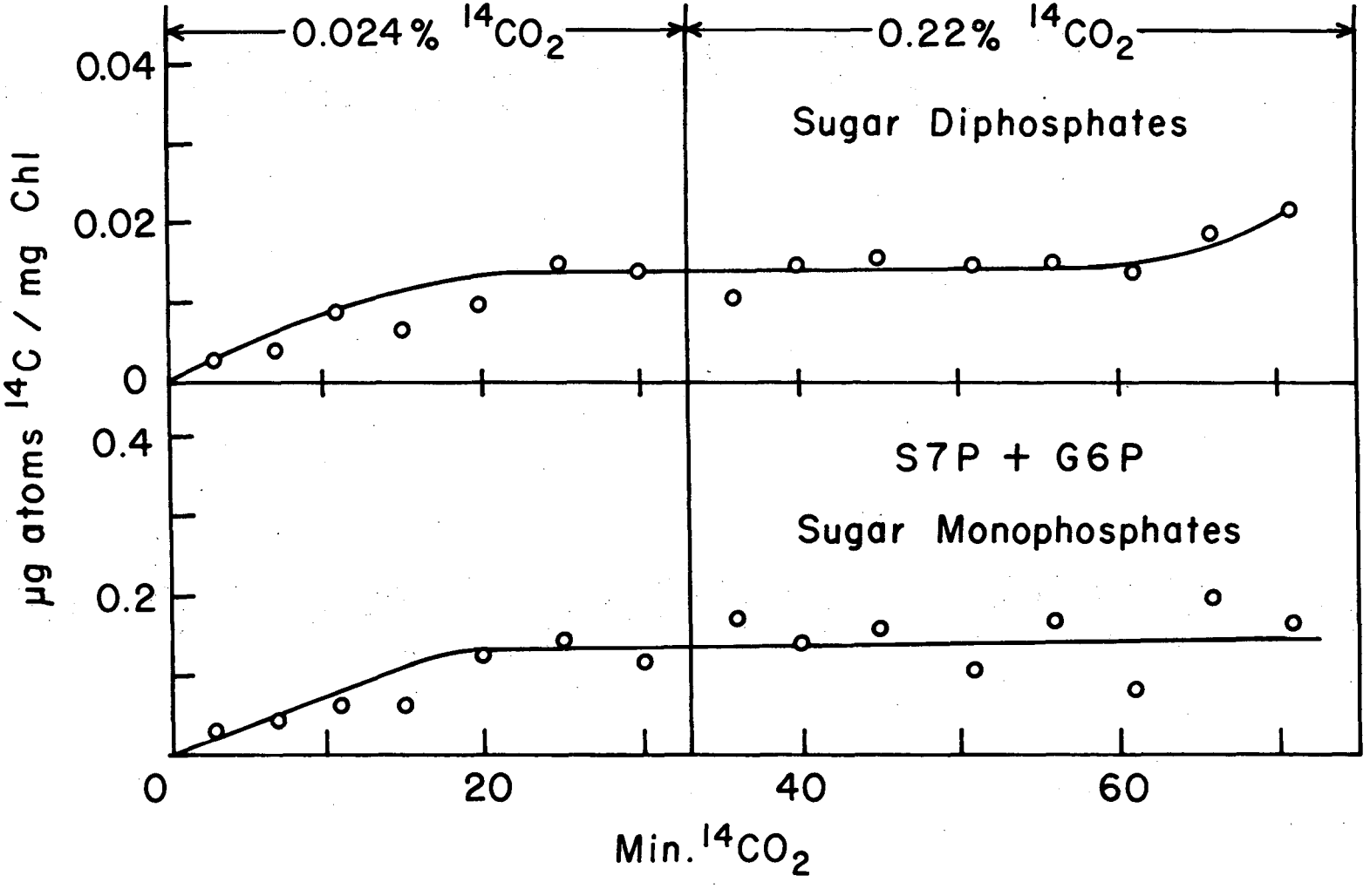


Figure 30. Incorporation of ^{14}C into PGA, glycerate, and serine by alfalfa leaflets during experiment A. The experimental conditions are given in Table 4.



XBL 763-5738

Figure 31. Incorporation of ^{14}C into sugar mono- and diphosphates by alfalfa leaflets during experiment A. The experimental conditions are given in Table 4.

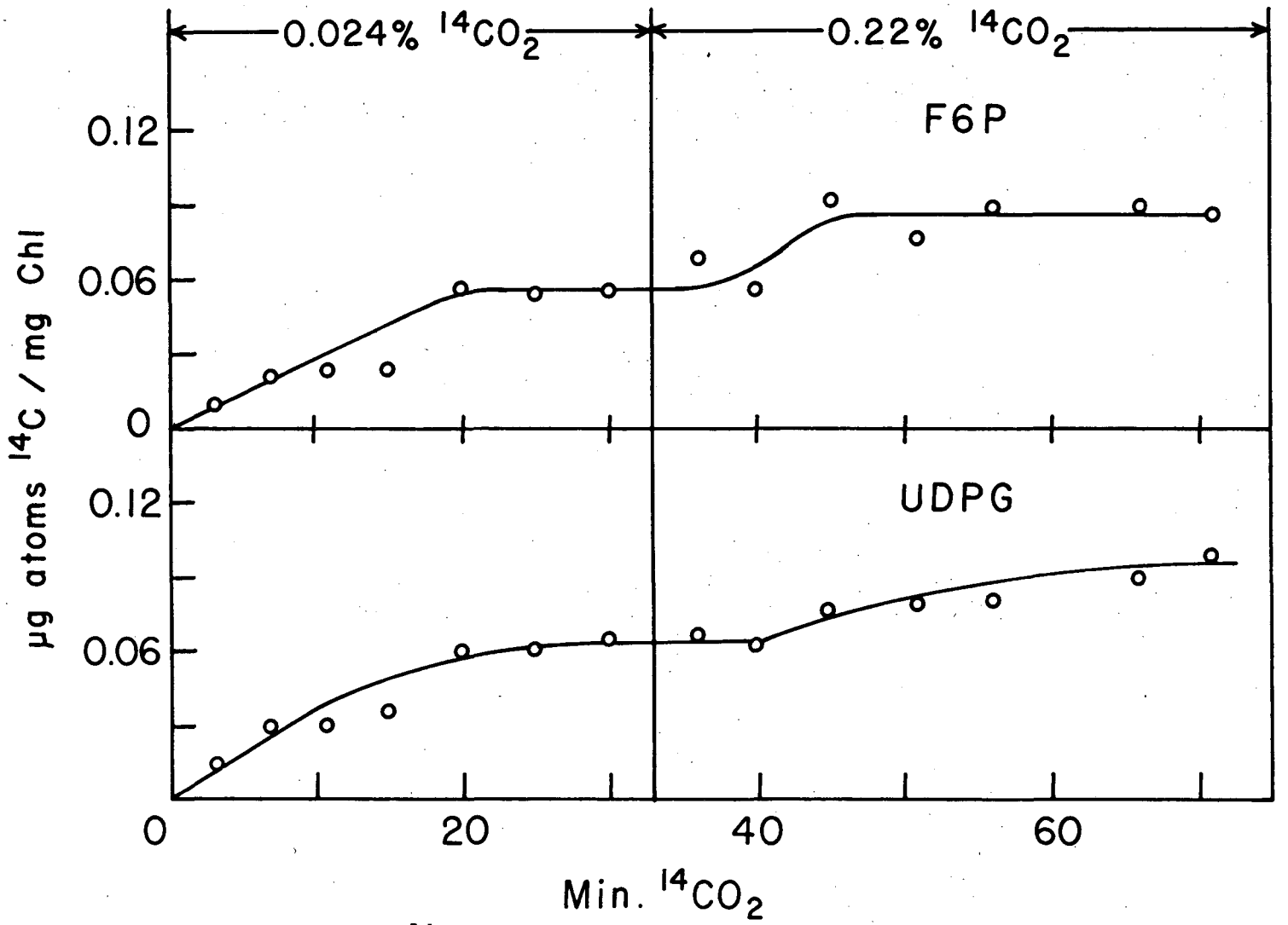


Figure 32. Incorporation of ^{14}C into F6P and UDPG by alfalfa leaflets during experiment A. The experimental conditions are given in Table 4.

XBL 763-5739

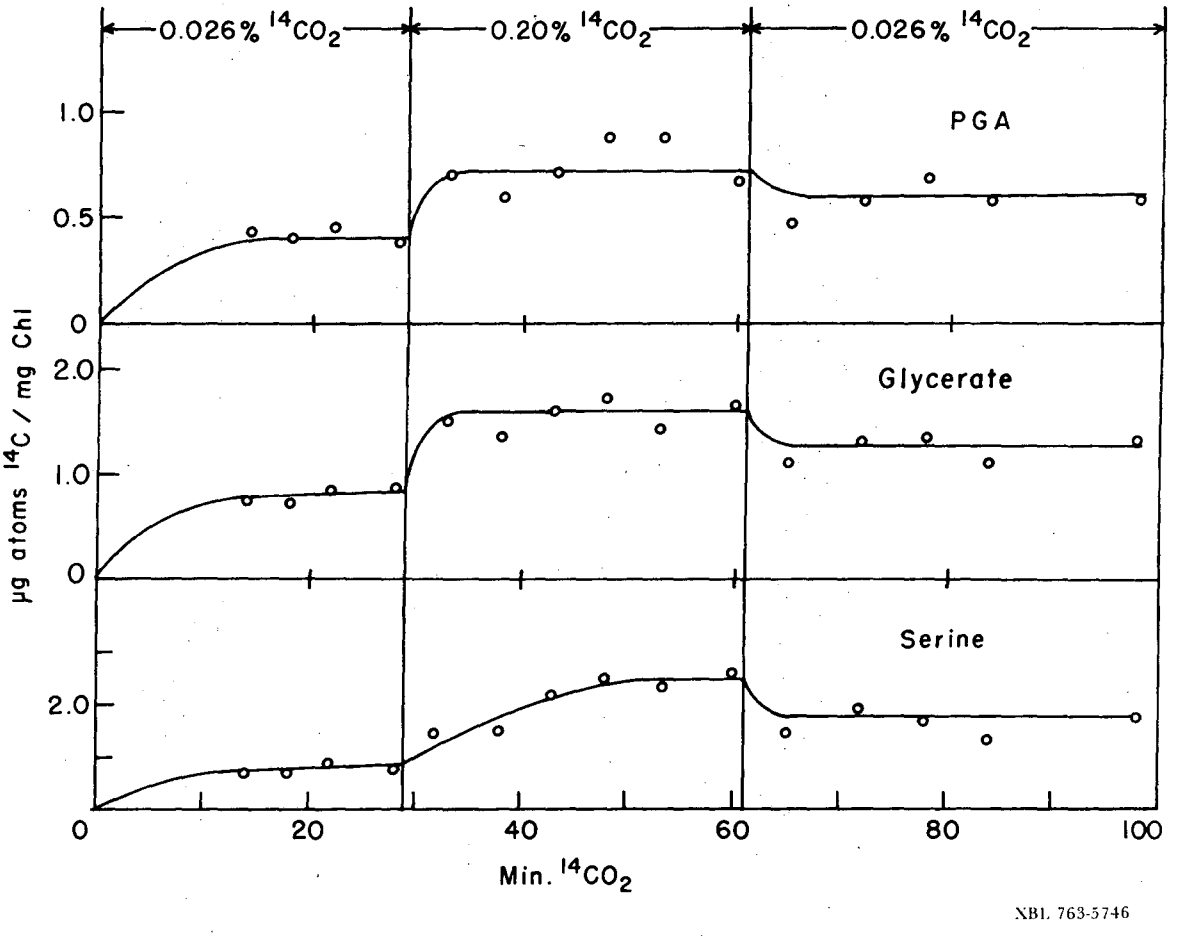


Figure 33. Incorporation of ^{14}C into PGA, glycerate, and serine by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

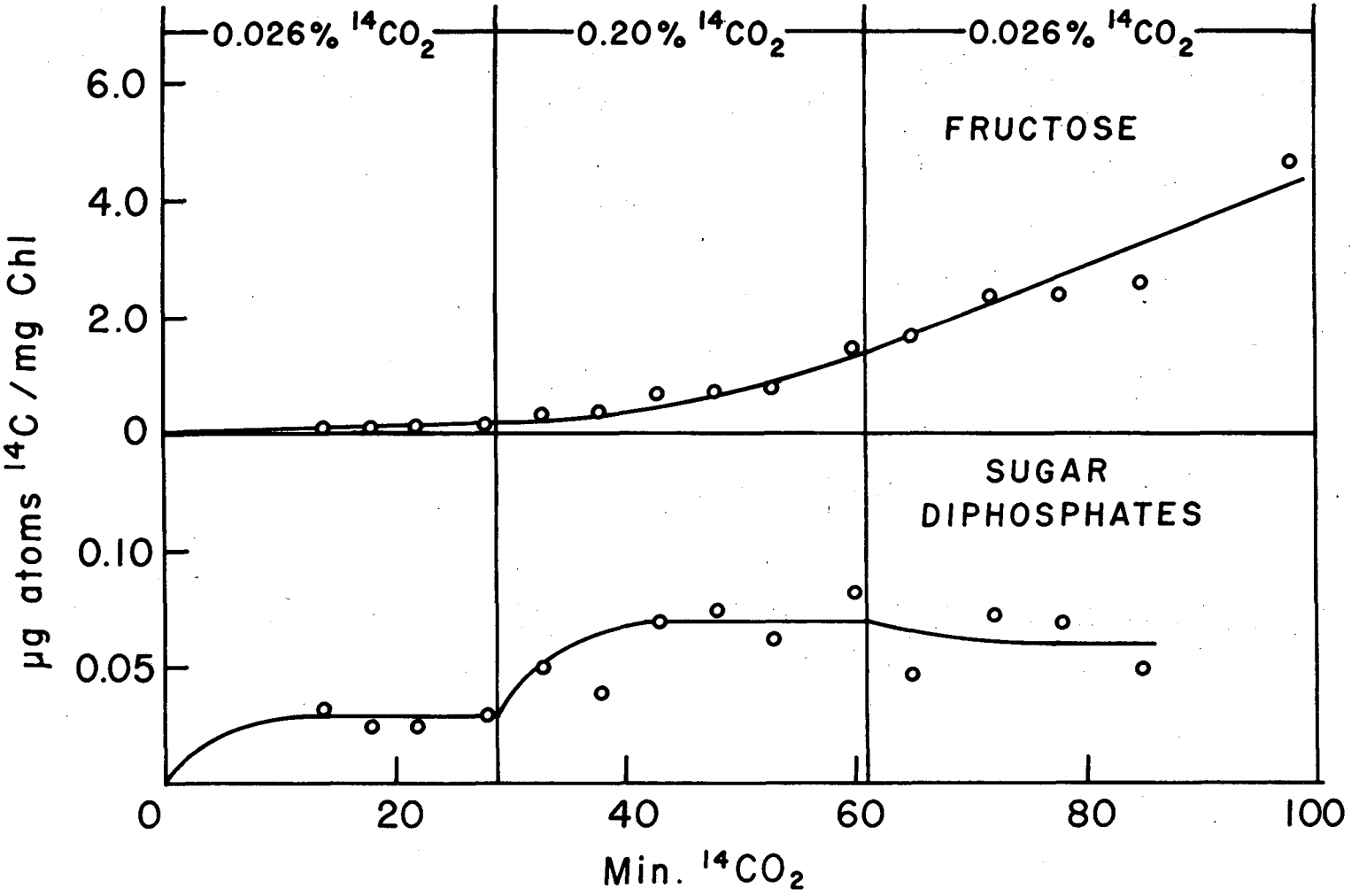


Figure 34. Incorporation of ^{14}C into fructose and the sugar diphosphates by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

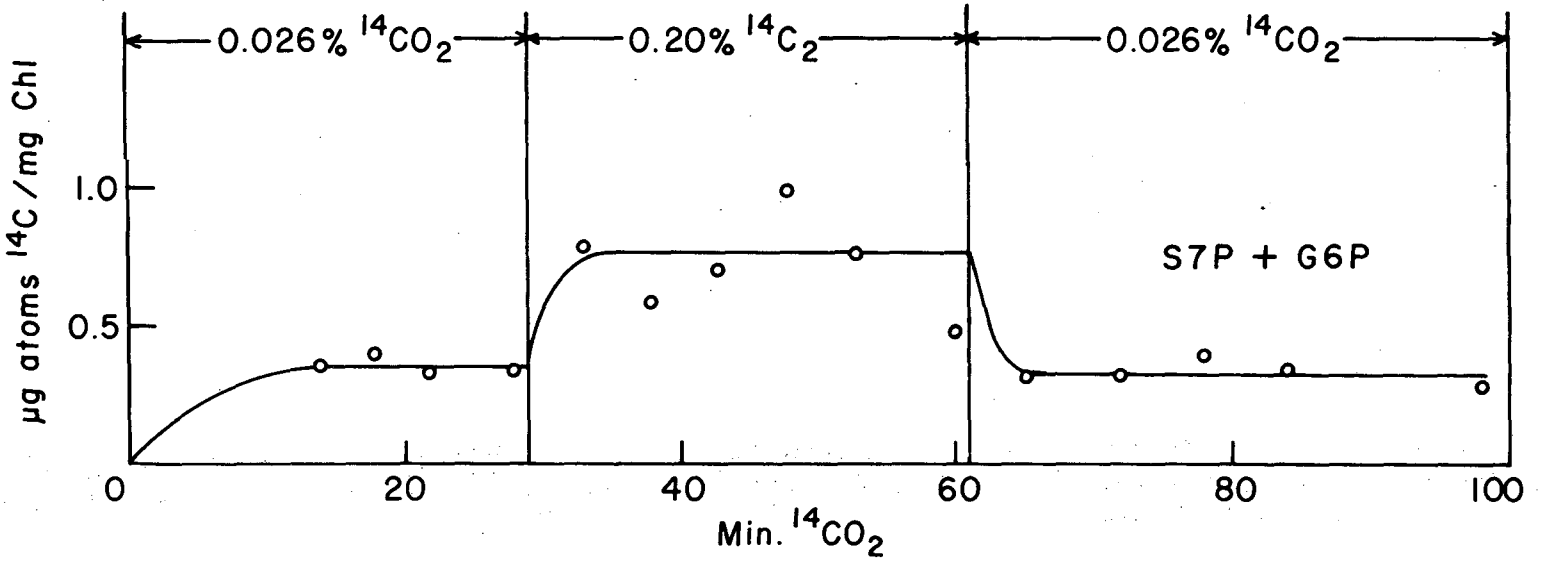


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Figure 35. Incorporation of ^{14}C into S7P + G6P by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

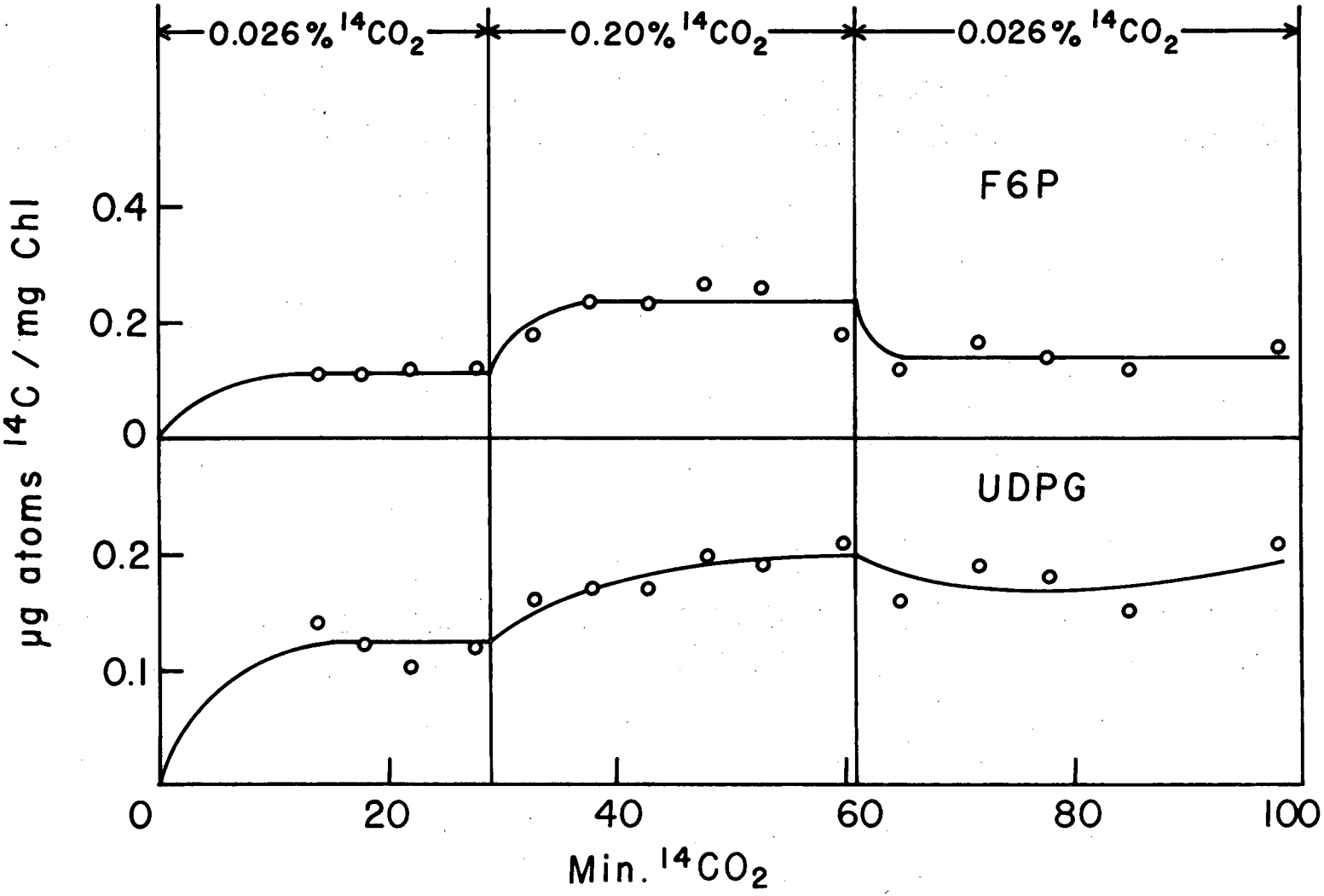
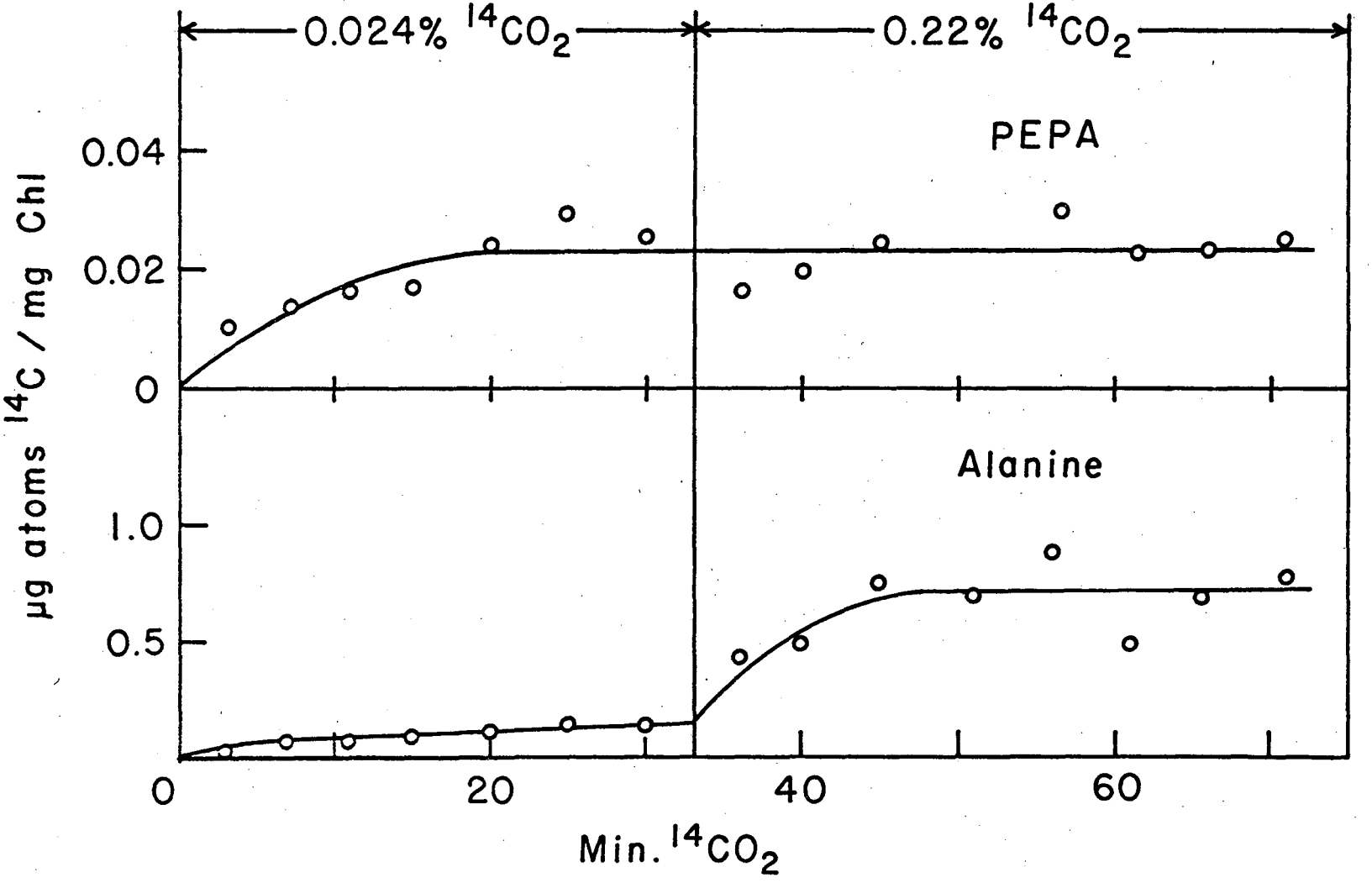


Figure 36. Incorporation of ^{14}C into F6P and UDPG by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

with half saturating $^{14}CO_2$. When the $^{14}CO_2$ concentration was increased, the steady state pool sizes of all those metabolites increased by approximately 100%. Returning the $^{14}CO_2$ to its original concentration resulted in a decline in their pool sizes (Figs. 33-36). S7P + G6P and F6P returned to their initial levels, while PGA and the sugar diphosphates reached equilibrium at concentrations approximately midway between those attained during the first two periods of photosynthesis. In experiment A, the increase in ^{14}C concentration observed for F6P could have been brought about by 3 seconds of the additional carbon flow indicated by the photosynthetic rate increase (Table 5). In experiment B, the increases observed in PGA, S7P + G6P, F6P and sugar diphosphates could be brought about by 42 seconds of the additional ^{14}C flow.

In experiments A and B, increased $^{14}CO_2$ concentration brought about an immediate increase in labeling of alanine (Figs. 37, 38). The labeling curve for PEPA in each experiment reflected that obtained for PGA in that particular experiment. This can be seen by comparing fixation into PGA shown in Fig. 30 to that shown for PEPA in Fig. 37, and PGA in Fig. 33 to that for PEPA in Fig. 38. In both experiments A and B the pool of recently fixed, photosynthetically active PEPA reached steady state during the initial period of photosynthesis with half saturating $^{14}CO_2$. When the level of carbon dioxide was increased during experiment A, the steady state concentrations of both PGA and PEPA did not change. In experiment B, the steady state concentrations of PEPA rose and fell with the CO_2 concentration as did PGA. Alanine labeling dropped sharply in experiment B when the $^{14}CO_2$ concentration was decreased (Fig. 38).

The labeling rates of the tricarboxylic acid cycle metabolites malate and citrate increased during both experiments when the $^{14}CO_2$



XBL 763-5743

Figure 37. Incorporation of ^{14}C into PEPA and alanine by alfalfa leaflets during experiment A. The experimental conditions are given in Table 4.

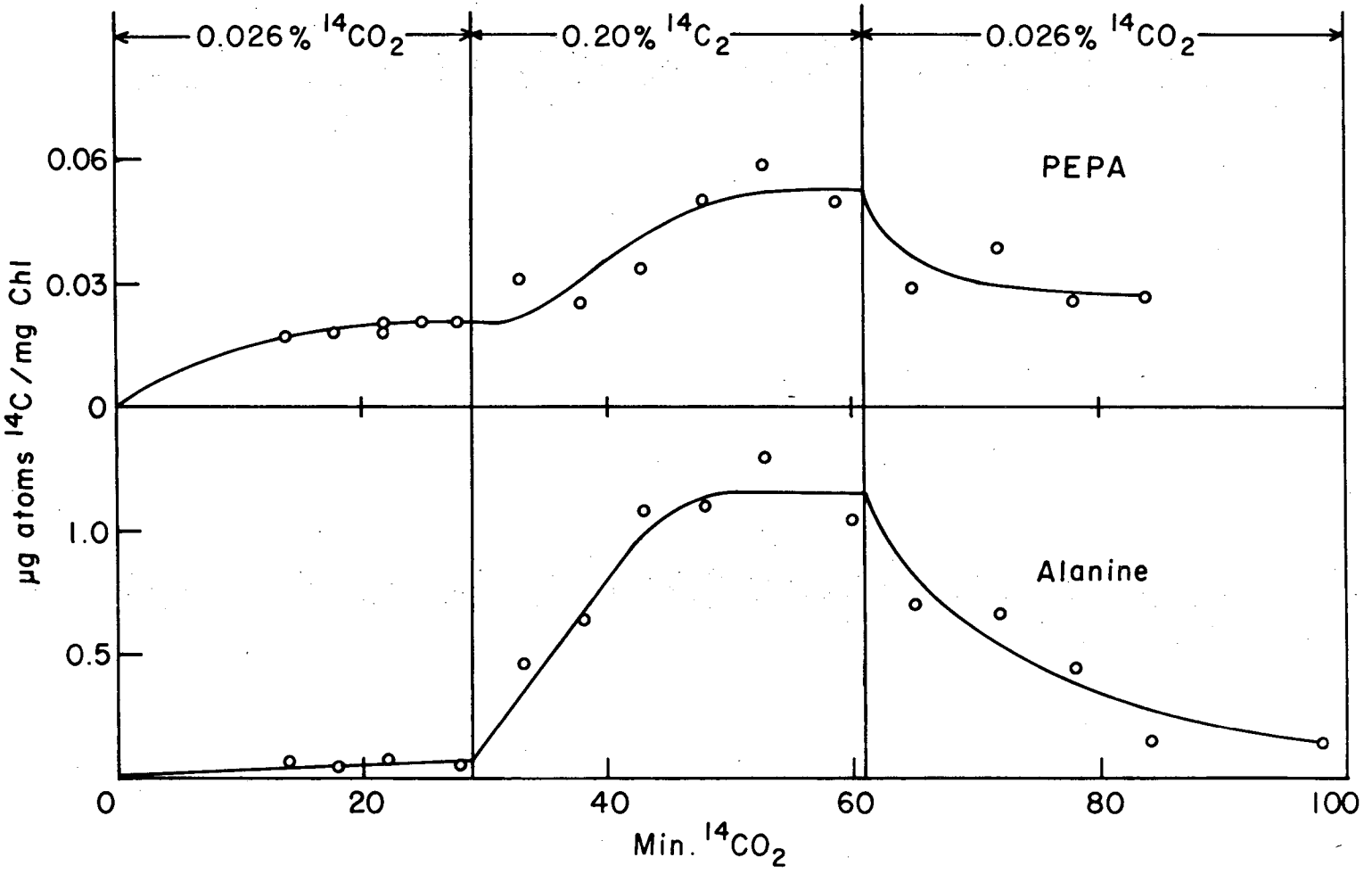


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Figure 38. Incorporation of ^{14}C into PEPA and alanine by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

level was raised (Figs. 39, 40). The citrate increases were especially sharp. When the $^{14}CO_2$ level was returned to half saturating during experiment B the level of label in both citrate and malate declined (Fig. 40). Aspartate and glutamate labeling curves (Figs. 41, 42) were similar to those obtained for citrate during experiments A and B. The labeling of aspartate and glutamate increased sharply when the $^{14}CO_2$ concentration was raised to saturating, and decreased when it was readjusted to half saturating. Citrate, aspartate, and glutamate labeling rates more than doubled when the $^{14}CO_2$ concentration was increased (Figs. 39-42).

Elevated $^{14}CO_2$ concentration brought about higher steady state levels of UDPG and F6P (Figs. 32, 36). Those two materials are probably the major precursors of sucrose (Zelitch 1971) and an increase in their photosynthetically active steady state concentrations would be expected to bring about increased sucrose production. Such an increase was observed (Figs. 28, 43). During the third period of experiment B (return to half saturating $^{14}CO_2$), the rate of labeling of sucrose decreased substantially (Fig. 43). This may partially be due to the approach of the large sucrose pool to ^{14}C saturation.

Labeling of the other free sugars, fructose and glucose, during experiments A and B is reported in Figs. 34 and 44. The glucose labeling curve from experiment B was similar to that shown for fructose in Fig. 34. Given the nonlinear labeling of these sugars in the nonperturbed steady state experiment described in Chapter I (Figs. 17, 22) the curves obtained are probably not shaped by the changing $^{14}CO_2$ levels.

The labeling curves for glycerate and serine were quite similar to that for PGA in experiment A (Fig. 30). PGA and glycerate reached ^{14}C saturation early in the low $^{14}CO_2$ concentration period (10-20 min),

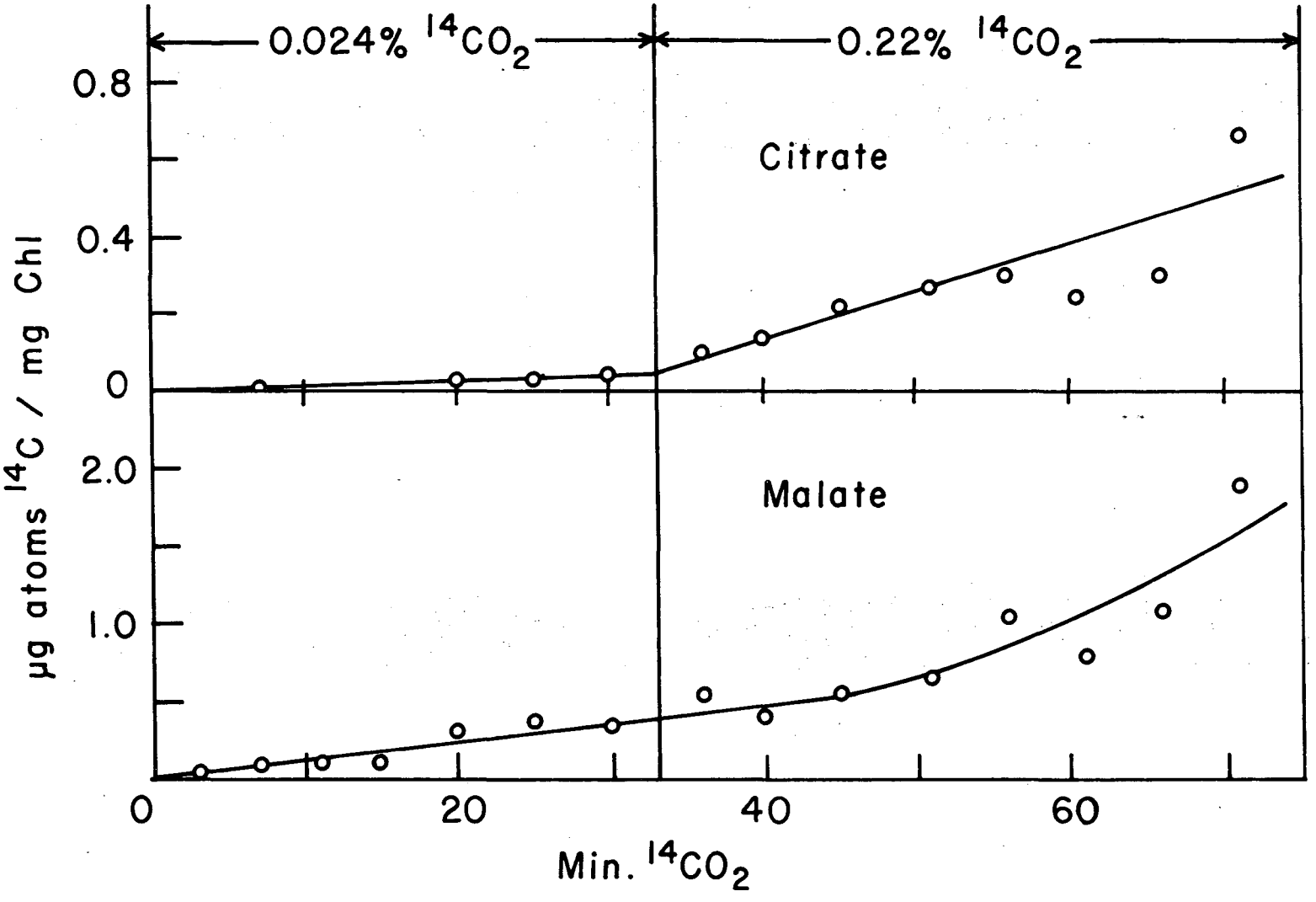

Figure 39. Incorporation of ^{14}C into citrate and malate during experiment A. The experimental conditions are given in Table 4.

XBL 763-5741

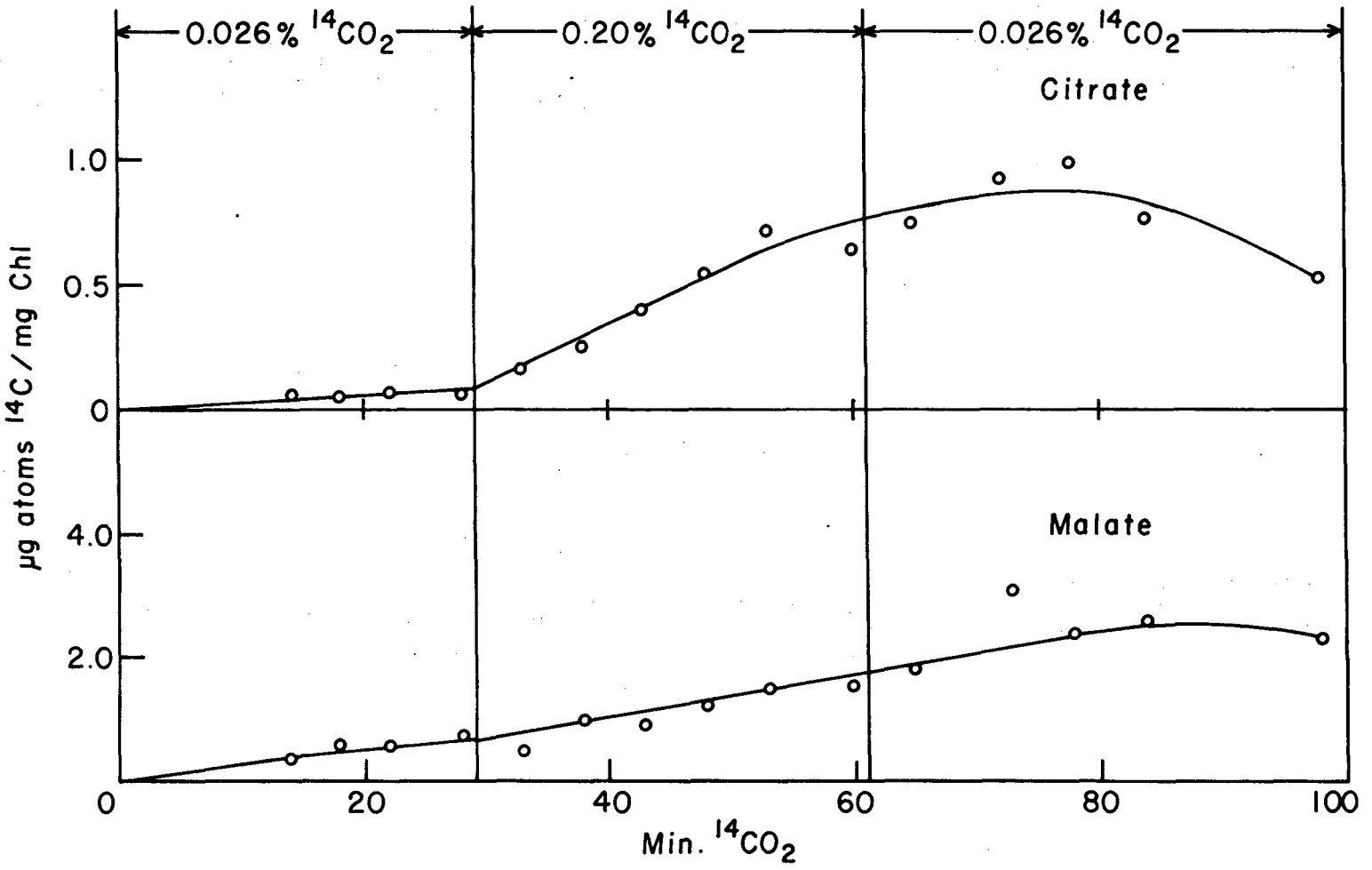

Figure 40. Incorporation of ^{14}C into citrate and malate by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

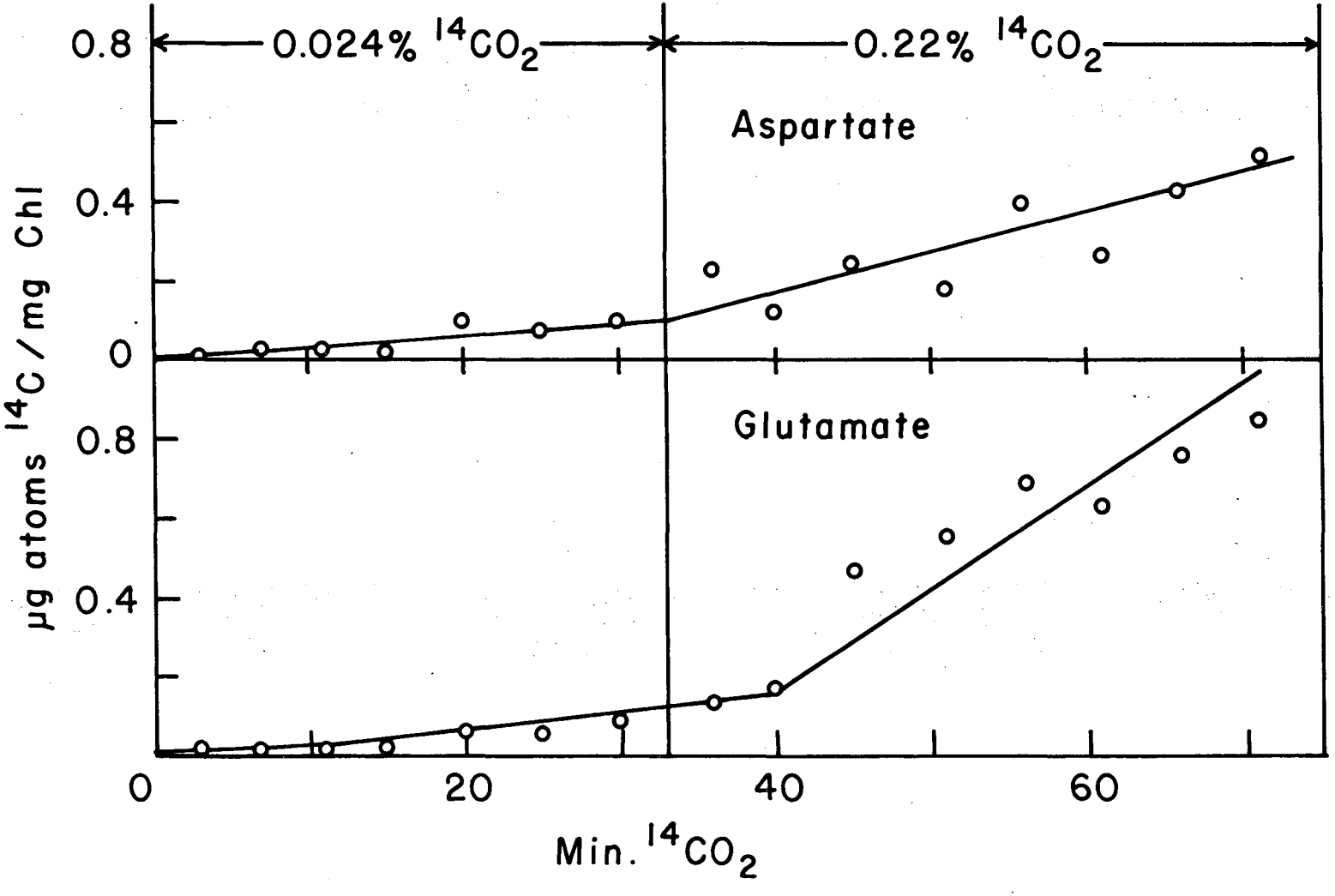

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Figure 41. Incorporation of ^{14}C into aspartate and glutamate by alfalfa leaflets during experiment A. The experimental conditions are given in Table 4.

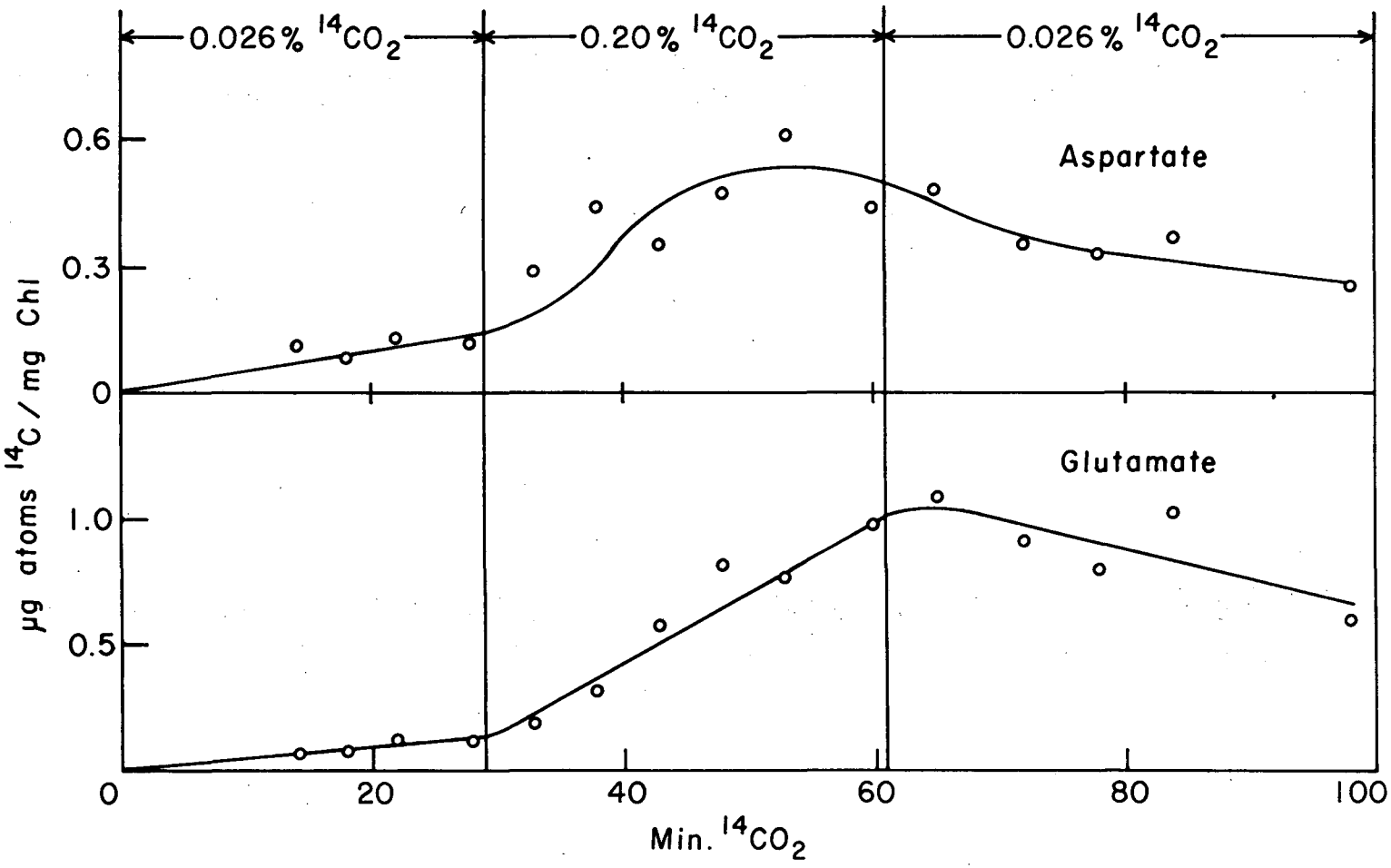

Figure 42. Incorporation of ^{14}C into aspartate and glutamate by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

XBL757-5333

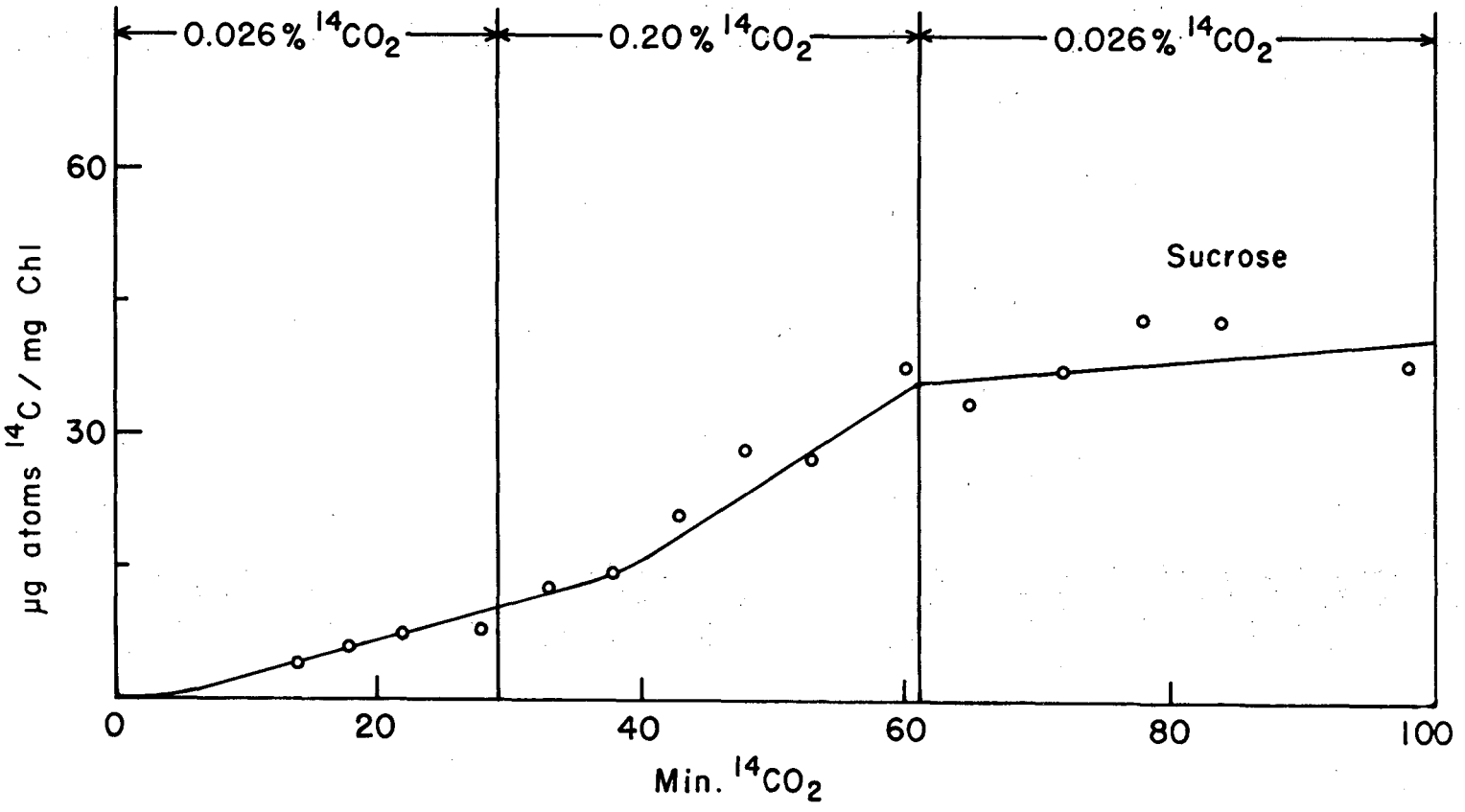

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Figure 43. Incorporation of ^{14}C into sucrose by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

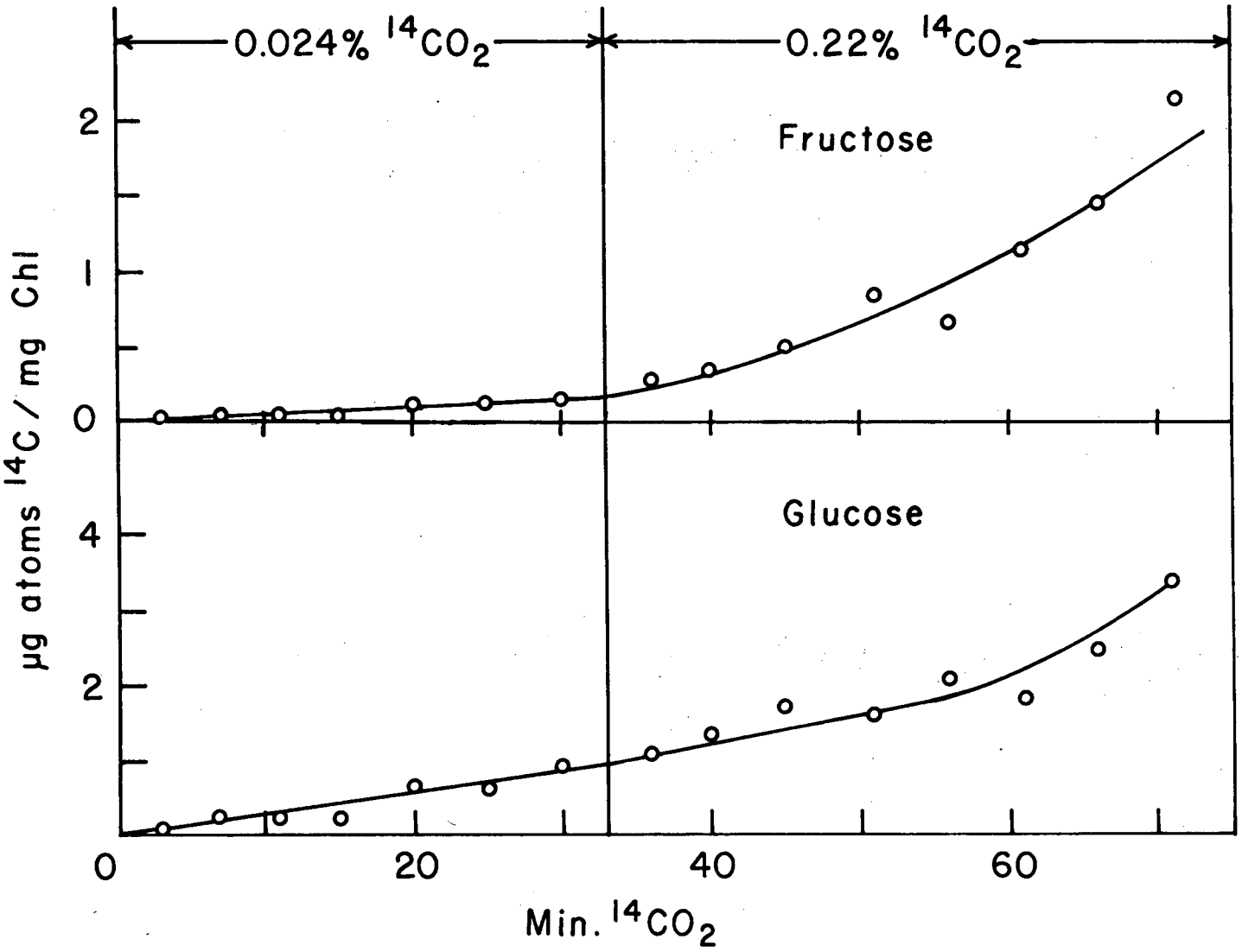

Figure 44. Incorporation of ^{14}C into fructose and glucose by alfalfa leaflets during experiment A. The experimental conditions are given in Table 4.

XBL 763-5740

serine reached saturation somewhat later (30 min). The steady state concentrations of recently fixed carbon found in the PGA, glycerate, and serine pools did not change when the $^{14}CO_2$ level was increased. The labeling curves obtained for glycine and glycolate in experiment A (Fig. 45) were quite different in form from those obtained for PGA, glycerate and serine (Fig. 30). Glycolate and glycine labeling increased with time during the first period of $^{14}CO_2$ photosynthesis and probably reached saturation after 20-30 min. Their level of labeling then dropped rapidly when the $^{14}CO_2$ concentration was increased, indicating that the pool sizes of photosynthetically active glycolate and glycine had decreased. New quite low levels of ^{14}C saturation were reached.

In experiment B, as in experiment A, the labeling curves obtained for PGA, glycerate, and serine were similar to each other (Fig. 33). PGA, glycerate and serine apparently reached ^{14}C saturation during the first period of photosynthesis with half saturating $^{14}CO_2$, the pool sizes of all three metabolites increased when the $^{14}CO_2$ concentration was changed to saturating. With a return to half saturating $^{14}CO_2$, all three materials declined to intermediate steady state concentrations. As in experiment A, the labeling curves for glycine and glycolate (Fig. 46) were of quite different form from those obtained for PGA, glycerate, and serine (Fig. 33). Glycolate and glycine labeling (Fig. 46) increased with time during the first period of photosynthesis with half saturating $^{14}CO_2$. When the $^{14}CO_2$ level was increased, the level of glycolate labeling decreased somewhat, while the level of label in glycine dropped sharply. These decreases indicate a change to smaller pools of photosynthetically active glycolate and

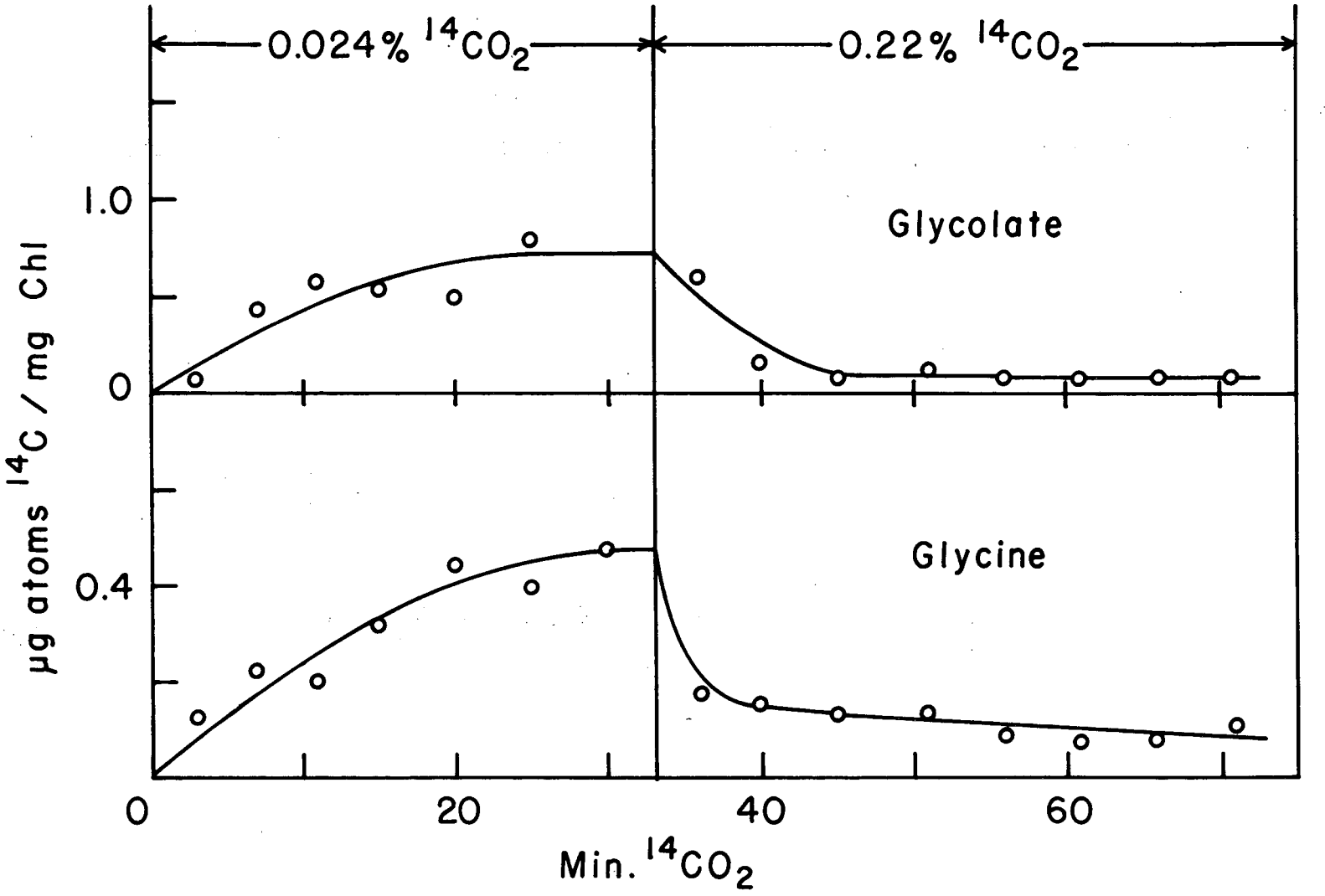

Figure 45. Incorporation of ^{14}C into glycolate and glycine by alfalfa leaflets during experiment A. The experimental conditions are given in Table 4.

XBL763-5714

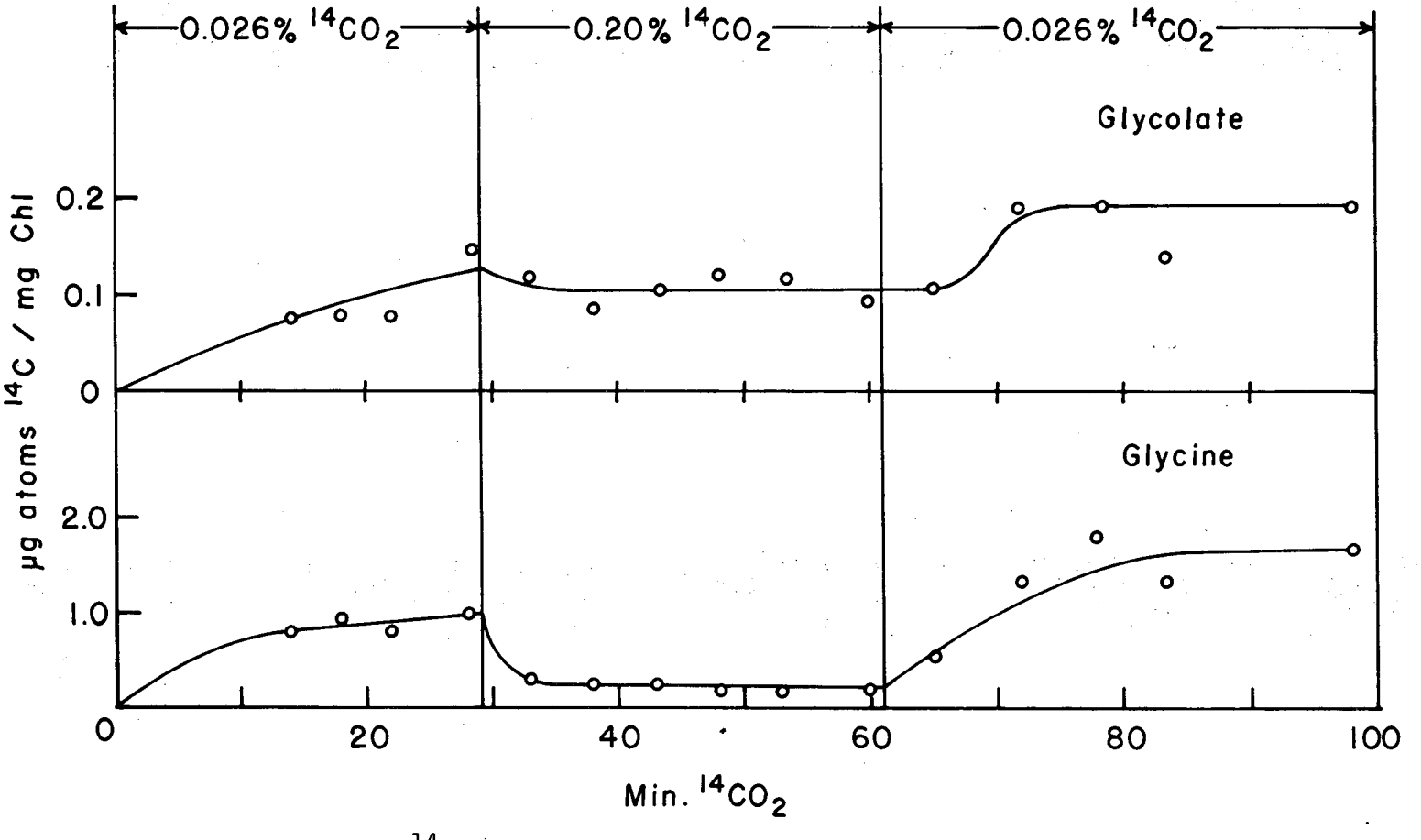

Figure 46. Incorporation of ^{14}C into glycolate and glycine by alfalfa leaflets during experiment B. The experimental conditions are given in Table 4.

XBL 763-5745

glycine. Both glycine and glycolate resumed the increase in labeling begun during the first period of photosynthesis when the $^{14}CO_2$ level was readjusted to half saturating (Fig. 46). Steady state concentrations higher than the level of labeling reached during the first period of photosynthesis with half saturating $^{14}CO_2$ were ultimately achieved.

The production of labeled glycine and serine during the initial period of photosynthesis with half saturating $^{14}CO_2$ was substantial in both experiments A and B. After 15 min of photosynthesis with $^{14}CO_2$ during experiment A, 6% of the soluble ^{14}C was present in glycine and 17% in serine. In experiment B, after 14 min of photosynthesis with half saturating $^{14}CO_2$, 8% of the soluble ^{14}C was found in glycine and 7% in serine. Label accumulation in glycolate was less substantial than in glycine + serine during both experiments A and B. After about 15 min of photosynthesis, 10% and 1% of the soluble label was found in glycolate in experiments A and B respectively.

Label accumulation in glycerate during the initial period of photosynthesis with half saturating $^{14}CO_2$ was quite high. After about 15 min of photosynthesis 10% and 8% of the soluble label was found in glycerate in experiments A and B respectively.

D. Discussion

1. Physiological Gas Exchange Parameters. The variation of alfalfa leaflet net photosynthetic rate with CO_2 concentration (Fig. 24) is analogous to results reported for several other species of higher plants by Gaastra (1959). The rate increase occurring in the region

of atmospheric CO_2 concentration probably reflects mainly the capacity of the CO_2 diffusion process (Gaastra 1962, Zelitch 1971) and increased rates of photosynthesis. Rate increases at much higher levels of CO_2 may reflect some suppression of photorespiration as well (Zelitch 1975a, Mahon et al. 1974). At CO_2 saturation the rate of photosynthesis is determined by some other factor such as the available light level or overall enzymatic carboxylation efficiency.

The CO_2 compensation points determined for El Unico alfalfa, 35 ppm at 18 C and 45 ppm at 23 C, are in agreement with a previous determination that alfalfa is a high compensation point species (Krenzer et al. 1975). The increase in CO_2 compensation point observed with temperature is expected for a C_3 plant (Joliffe and Tregunna 1968).

The curve obtained for alfalfa photosynthesis as a function of oxygen concentration (Fig. 25) is similar to that found for soybean (another legume) by Forrester et al. (1966a). The photosynthetic rate increases observed on decreasing the oxygen concentration from approximately 21% to 2% were 50% at 23 C and 32% at 17 C. As mentioned previously, the net photosynthetic rates of C_3 species are strongly inhibited by an increase in oxygen concentration of from about 2% to 21% (air level CO_2, high illuminance). This effect has been related to the measurable photorespiration (Zelitch 1971) and possible O_2 inhibition of photosynthesis (Chollet and Ogren 1975) which occurs in those plants. Species with high rates of photorespiration show large increases (above 33%) in photosynthetic rates at 2% O_2 relative to 21% O_2. Species with low rates of photorespiration show only minor increases (0-6%) in photosynthetic rates (Zelitch 1971). Hence the rate increases observed for alfalfa are in accord with its classification as a C_3 plant. It can be noted that 1/16 of the rate increase observed at 17 C may be

accounted for by repression of dark respiration at the lower O_2 concentration.

The rate of photorespiration estimated to have been occurring in the alfalfa leaflets at 17 C (16 μmoles CO_2/hr·mg Chl, 1.7 times dark respiration) is comparable to extrapolation data tabulated by Zelitch (1971). Zelitch reports photorespiration/dark respiration ratios of 2.1-4.9 at 25-26 C. The lower ratio found for alfalfa is understandable given the lower temperature of my experiment (Jackson and Volk 1970).

In summary, the results of the gas exchange parameter investigations support classification of alfalfa as a C_3 plant. The observed CO_2 compensation point, photosynthetic rate increase on lowering the oxygen concentration, and the extrapolated value of the photosynthetic rate vs. CO_2 concentration curve all indicate substantial photorespiration was probably occurring at 17 C and 23 C. The photosynthetic rate increases observed on lowering the O_2, or raising the CO_2 concentration, relative to that in the air are in accord with the idea that the crop productivity of a given variety of alfalfa could be increased by atmospheric manipulation.

2. $^{14}CO_2$ Fixation Experiments. The photosynthetic rates observed at half saturating $^{14}CO_2$, when converted to units of mg CO_2/hr·dm^2 (Chapter I), were comparable to values reported in the literature (Wolf and Blazer 1972, Delaney et al. 1974, Pearce et al. 1968). The photosynthetic rate increase observed in experiments A and B when the $^{14}CO_2$ concentration was increased, approximately 100%, is of the order expected from the data presented in Fig. 24. That increase is similar to the extent of rate increase noted by Gaastra (1959) at similar light levels in several other higher plant species.

In both experiments A and B the rate increase observed on increasing the $^{14}CO_2$ concentration appeared to be accounted for more by increased labeling of insoluble products than soluble products (Table 5). Combining of data on many individual metabolites under headings such as "soluble" and "insoluble" products involves some dangers. As photosynthesis with $^{14}CO_2$ proceeds, some soluble metabolites become saturated with ^{14}C and this will decrease the slope of the line representing $^{14}CO_2$ uptake into solubles. The slope of that line will therefore not reflect the full increase of ^{14}C flow through the pool of soluble materials. Furthermore, any rate increase in labeling of insolubles can occur only pursuant to increased ^{14}C flow through soluble pools. A cautious interpretation of the data presented in Table 5 as regards rate increases in soluble and insoluble products is simply that both pools participate approximately equally in the overall rate increase. The extremely low rate of labeling of soluble products during the final 0.026% $^{14}CO_2$ period of experiment B (Table 5) is due to full or near ^{14}C saturation of many soluble metabolites (especially sucrose, Fig. 43) which occurred during the first 61 min of photosynthesis with $^{14}CO_2$, and to a decrease of the absolute levels of labeling of some soluble metabolites during the final 37 min (Figs. 33-36, 38, 40, 42). Continued $^{14}CO_2$ incorporation, and increased insoluble labeling, during the third period of experiment B (Table 5), indicates that carbon flow through the soluble pools was still occurring at about the same rate as during the initial 0.026% $^{14}CO_2$ period.

In experiment A increased $^{14}CO_2$ concentration brought about an increased pool size of F6P (Fig. 32) but other identified RPP metabolites did not increase in pool size (S7P + G6P and sugar diphosphates, Fig. 31). In experiment B pool sizes of PGA (Fig. 33),

sugar diphosphates (Fig. 34), S7P + G6P (Fig. 35) as well as that of F6P (Fig. 36) increased. While the exact effects of higher CO_2 are somewhat different in each experiment, the same general observation can be noted: Only minor absolute changes occurred in the size of the photosynthetically active pools of RPP cycle intermediates when the $^{14}CO_2$ concentration was changed from half saturating to saturating. In both experiments the changed RPP cycle pool sizes could be brought about by less than 0.75 min of the additional ^{14}C flow. A large absolute increase in carbon flow through the RPP cycle, therefore, occurred with only a small absolute change in the amounts of recently fixed photosynthetically active carbon accumulating in those metabolites. This testifies to the well balanced nature of the RPP cycle and the carbon flow take offs leaving it. Apparently withdrawal of ^{14}C from the RPP cycle for use in leaf metabolism increased almost as rapidly as did the $^{14}CO_2$ influx into that cycle. Only very small relative differences between $^{14}CO_2$ influx and ^{14}C withdrawal rates in the two experiments could account for the pool size effects observed. Such differences may have been reflections of minor differences in the physiological state of the leaf material used. The experiment A results where a large increase in $^{14}CO_2$ incorporation rate occurred with almost no change in RPP cycle pool sizes are suggestive of a regulatory interaction between $^{14}CO_2$ transport into the chloroplast and ^{14}C-metabolite transport out of it. In nonkinetic (single time point) $^{14}CO_2$ fixation experiments at differing levels of carbon dioxide, Snyder and Tolbert (1974) and Osmond and Bjorkman (1972) reported greater absolute ^{14}C fixation into RPP intermediates at elevated CO_2 levels. Those experiments included short term exposure of C_3 higher plants other than alfalfa to $^{14}CO_2$. Without sampling as a function of

time, it is impossible to know to what extent their results represent changes in ^{14}C saturation levels and to what extent they represent differences in ^{14}C flow rates.

In the following paragraphs I will discuss data obtained on photosynthetic carbon flow to the TCA cycle, amino acid production, sucrose, and metabolites usually associated with the glycolate pathway. In analyzing the perturbed steady state kinetic labeling curves, conclusions about carbon flow will be based upon observation of the simultaneous effects of the $^{14}CO_2$ concentration change on labeling of several metabolites. The rate of labeling of a metabolite prior to saturation, and its steady state pool size (level of labeling at ^{14}C saturation), depend on the rates of reactions following and preceding formation of that metabolite. Examination of the simultaneous changes occurring in several metabolites in a sequence allows for clearer interpretation of the overall effects of a perturbation than does examination of labeling curves for only one metabolite at a time. This approach was reviewed by Bassham (1973). The availability of kinetic data, that is, of ^{14}C incorporation data as a function of time, should aid in distinguishing between changes in metabolite labeling rates and steady state concentrations. Such multipoint data should be inherently more reliable, and more clearly interpretable, than data from single time point $^{14}CO_2$ fixation experiments.

In discussion of carbon flow to and from TCA cycle metabolites, some reference will be made to the general indications of the data presented in Chapter I when analyzed in conjunction with the data from experiments A and B. The conditions of the experiment described in Chapter I, and the photosynthetic rate observed, are quite similar to those of the half saturating $^{14}CO_2$ periods of experiments A and B

(Tables 4 and 5). Conditions under which that experiment, now labeled experiment C for convenience, was conducted are presented in Table 6. The approximate amounts of label found in several metabolites during experiments A, B, and C after 28-30 min of photosynthesis with half saturating $^{14}CO_2$ are presented in Table 7. Fixation into the various metabolites was generally of the same order in the three experiments. The similarity of the extents of labeling (Table 7), and basic conditions of the three experiments supports the use of experiment C data to aid in interpreting the experiments A and B labeling curves. The labeling curves obtained in experiment C are to some extent control curves (no perturbation) for the perturbed steady state experiments A and B.

The increased labeling of alanine during the high $^{14}CO_2$ concentration period of experiments A and B is emphasized by comparison of the curves presented for that metabolite in Figs. 37 and 38 to the gently rising curve presented in Fig. 16. Alanine label content at the close of the saturating $^{14}CO_2$ periods (Figs. 37, 38) was approximately 7 times greater than at the close of experiment C (Fig. 16). This strongly suggests that alanine pool size, as well as its labeling rate, increased during the high $^{14}CO_2$ period. The decrease in alanine labeling occurring during the final period of experiment B (Fig. 38) indicates a return to a smaller pool of photosynthetically active material. The increased labeling rate of the tricarboxylic acid cycle metabolite citrate when the $^{14}CO_2$ level was raised (Figs. 39, 40) led to ^{14}C-citrate concentrations an order of magnitude greater than the steady state concentration reached in experiment C (Fig. 16). The curves for citrate and malate from experiment C (Fig. 16, 18) suggest that without the increase in $^{14}CO_2$ concentration, the ^{14}C level of

Table 6. Conditions of Experiment C and Photosynthetic Rate Observed (from Chapter I)

$^{14}CO_2$ concentration (%)	0.027 ± 0.003
Time (min)*	0 - 48
Light level (ft-c)	3600
Oxygen concentration (%)	20 ± 1
Temperature (C)	18 ± 1
$^{14}CO_2$ specific radioactivity (μCi/μmole)	16.6
Observed photosynthetic rate (μg atoms ^{14}C/hr·mg Chl)	79

* from initiation of photosynthesis with $^{14}CO_2$.

Table 7. Amounts of ^{14}C Found in Several Metabolites After 28-30 min of Photosynthesis with Half Saturating $^{14}CO_2$*

	Experiment		
	A	B	C
PEPA	0.03	0.02	0.05
Alanine	0.13	0.05	0.12
Citrate	0.04	0.06	0.07
Malate	0.4	0.8	1.0
Aspartate	0.10	0.12	0.24
Glutamate	0.10	0.12	0.10

* All values in units of μg atoms ^{14}C/mg Chl.

Data from Figs. 16, 18-20, 37-42.

those metabolites would have soon reached saturation at low level CO_2. The curves in Figs. 39 and 40 probably reflect increased sizes of photosynthetically active citrate and possibly malate pools under conditions of elevated CO_2, as well as increased flow of recently fixed carbon into those TCA cycle metabolites. The labeling curves from experiments A and B appear to indicate that aspartate and glutamate pool sizes as well as labeling rates also increased under saturating $^{14}CO_2$ (Figs. 41, 42) and then declined when the $^{14}CO_2$ concentration was changed to half saturating. Steady state concentrations obtained for pools of photosynthetically active aspartate and glutamate from experiment C were 0.24 and 0.13 μg atoms ^{14}C/mg Chl, respectively. Under conditions of high CO_2 in experiments A and B, the ^{14}C found in those metabolites was approximately 2-7 times those values (Figs. 41, 42). Madsen (1974) previously found elevated total pool sizes of malate and citrate in tomato leaves from plants grown at CO_2 levels above that of air. The largest increases were noted after several hours of photosynthesis had occurred.

The data on PEPA, alanine, malate, citrate, glutamate and aspartate from experiments A, B, and C, combined with observed increased total photosynthetic rate and insolubles labeling rate (probably polysaccharides, proteins, etc.) when the $^{14}CO_2$ level was raised are consistent with the paths of carbon flow I suggested in Fig. 23. My conclusions about the effects of the transfer from half saturating to saturating $^{14}CO_2$ on carbon flow along the paths shown in Fig. 23 are as follows: When the $^{14}CO_2$ level is increased from half saturating to saturating the rate of photosynthesis approximately doubled (Table 5, Figs. 26, 27). Carbon flowed into and out of the RPP cycle at an increased rate. The flow of carbon from the RPP cycle for use in synthesis of TCA cycle intermediates increased (Figs. 39, 40). More ^{14}C material therefore

flowed to and through the PEPA pool. The PEP-carboxylase reaction was presumably stimulated by the increased level of its substrate $^{14}CO_2$ and produced oxaloacetate at an increased rate. The increased level of $^{14}CO_2$ also appears to have brought about (perhaps indirectly) an activation of pyruvate kinase. That activation is suggested by the abrupt rise in alanine labeling and pool size which occurred in experiments A and B when the CO_2 level was increased (Figs. 37, 38). Labeled alanine presumably was produced by transamination of pyruvate produced from PEPA by the pyruvate kinase reaction. Increased alanine labeling, and pool size, therefore probably indicates an increase in the size of the photosynthetically active ^{14}C-pyruvate pool. Activation of pyruvate kinase under the conditions of elevated $^{14}CO_2$ is especially strongly supported by the data of experiment A where increased alanine labeling (Fig. 37), and presumably pyruvate level, was observed when the level of photosynthetically active labeled PEPA did not increase. In experiment A, the increased withdrawal of PEPA carbon at saturating $^{14}CO_2$ (for OAA and pyruvate synthesis) apparently balanced immediately the increased flow of ^{14}C into that pool and the level of photosynthetically active PEPA did not rise (Fig. 37). In experiment B, additional withdrawal of ^{14}C-PEPA did not initially balance the added ^{14}C influx. The PEPA rose under the condition of saturating $^{14}CO_2$ to a higher steady state level (Fig. 38). The observed increases in malate and citrate labeling and probably pool sizes (Figs. 39, 40) support the idea that increased ^{14}C flow into the TCA cycle occurred through the anapluerotic PEP-carboxylase and malic enzyme reactions. That increased flow would occur in direct response to elevated substrate levels ($^{14}CO_2$, pyruvate, and in experiment B, PEPA). Some enzyme activation may also have occurred. The increased rate of formation and pool size, of

photosynthetically active citrate (Figs. 39, 40) indicates a greater flow of pyruvate into the TCA cycle through formation of acetyl CoA which condenses with oxaloacetate. The alanine data (Figs. 37 and 38) indicates that the proposed increased withdrawal of ^{14}C-pyruvate mediated by malic enzyme, and also for use in producing acetyl CoA, did not quite match the increased flow of ^{14}C which entered the pyruvate pool through the pyruvate kinase reaction. Increased carbon flow through the PEP-carboxylase and malic enzyme mediated reactions, and increased entry of acetyl CoA into the TCA cycle, accounts for the observed increases in aspartate and glutamate labeling and pool sizes (Figs. 41 and 42). Increased synthesis of aspartate and glutamate using recently fixed carbon then presumably led to greater rates of synthesis of other amino acids of which those two are precursors (Fowden 1965). Ultimately, increased protein synthesis would occur. That was included in, and consistent with, the observed greater rate of synthesis of insolubles at elevated $^{14}CO_2$ (Table 5).

In summary, the overall picture of carbon flow consistent with Fig. 23 and Figs. 37-42 is of ^{14}C flow to and through the TCA cycle metabolites participating in the general photosynthetic rate increase observed at saturating vs. half saturating $^{14}CO_2$. Decreased levels of labeling and pool sizes of PEPA and alanine (Fig. 38), malate and citrate (Fig. 40), and of glutamate and aspartate (Fig. 42) observed in experiment B on returning to half saturating $^{14}CO_2$ indicate that the effects of the $^{14}CO_2$ concentration change were reversible. Decreased $^{14}CO_2$ concentration apparently resulted in a return to lower levels of tracer carbon flow through the TCA cycle and to amino acid production; with a concomitant drop in the pool sizes of labeled photosynthetically active PEPA, alanine, citrate, malate, aspartate

and glutamate.

Sucrose was by far the major labeled soluble product in experiments A and B (Table 8). Its labeling accounts for 29% of the total photosynthetic rate during the first period of experiment A, and 35% of that rate during the first period of experiment B. The absolute labeling rate of sucrose increased greatly under the condition of saturating $^{14}CO_2$, but its rate of labeling as a percent of the total fixation rate increased by only 11% in experiment A and 2% in experiment B (Table 8). The general conclusion that can be drawn is that sucrose labeling participated in the general photosynthetic rate increase, and its labeling accounted for a slightly greater proportion of the total fixation at high CO_2.

Table 8. Rate of Increase of ^{14}C in Sucrose*

Experiment A	First Period	Second Period	Third Period
$^{14}CO_2$ concentration (%)	0.024	0.22	-
^{14}C incorporation into sucrose			
μg atoms/mg Chl·hr	16	42	-
% of total photosynthetic rate	29	40	-
Experiment B			
$^{14}CO_2$ concentration (%)	0.026	0.20	0.026
^{14}C incorporation into sucrose			
μg atoms/mg Chl·hr	25	55	10
% of total photosynthetic rate	35	37	16

* Data taken from Figs. 26-28, 43.

The last group of metabolites studied includes glycolate, glycine, serine and glycerate. Formation of those metabolites has generally been associated with the glycolate pathway sequence glycolate↣glycine→serine↣glycerate (Tolbert 1973a and b). The two amino acids glycine and serine are essential precursors for proteins, C_1-biosynthesis and porphyrins (Tolbert 1973b). Recent data led me to conclude (Chapter I) that in alfalfa photosynthesizing with half saturating $^{14}CO_2$ glycerate was formed directly from 3-PGA through the reaction mediated by 3-phosphoglycerate phosphatase. The 3-phosphoglycerate phosphatase

reaction is the first step in a proposed alternative to the glycolate pathway for synthesis of glycine and serine (Randall et al. 1971). That alternative path consists of the sequence PGA→glycerate→→serine→ glycine. Following hydrolysis of PGA by 3-phosphoglycerate phosphatase the glycerate→→serine→glycine conversions are proposed to proceed by reversal of the glycolate pathway reactions. The alternative path does not depend upon glycolate synthesis in order to produce glycine and serine. It avoids the loss of photorespiratory CO_2 which occurs during the glycine→serine step of the glycolate pathway (Tolbert 1973a and b). The data obtained from experiments A and B indicates the operation in alfalfa of part of the glycolate pathway (Tolbert 1973a) and part of the alternative pathway (Randall et al. 1971).

An elevated concentration of $^{14}CO_2$ (saturating) brought about decreased levels of label in glycine and glycolate (Figs. 45, 46). Serine and glycerate levels of labeling on the contrary remained constant in one experiment and increased in the other (Figs. 30, 33). The serine and glycerate labeling curves were strikingly similar to the curve obtained for PGA in the same experiment (Figs. 30, 33). In both experiments A and B, glycine appeared to be responding to changes in the pool size of photosynthetically active glycolate. Serine and glycerate, however, seemed in both experiments to respond to the size of the PGA and not the glycolate pool. The data can best be interpreted as indicating glycine production from glycolate by means of the glycolate pathway, and synthesis of glycerate and serine predominantly from PGA by means of the alternative pathway initiated by 3-phosphoglycerate phosphatase.

Although serine appeared to be synthesized predominantly from PGA, some carbon flow to serine from glycolate may have been occurring at

half saturating $^{14}CO_2$ given the evidence I presented earlier for the occurrence of photorespiration in alfalfa. Alfalfa was determined to have a high CO_2 compensation point, its photosynthetic rate increased on lowering the external oxygen concentration, and the extrapolated value of its photosynthetic rate vs. CO_2 concentration curve was quite negative. As was mentioned in the Introduction, carbon flow from glycolate to serine could contribute to that photorespiratory CO_2 release. It is possible, however, that alfalfa photorespiratory CO_2 release occurred mainly by the mechanism discussed by Zelitch (1975b) which does not involve formation of serine from glycolate.

The data indicate carbon flow occurring in experiments A and B was as follows: With the initiation of photosynthesis with half saturating $^{14}CO_2$, flow of recently incorporated tracer carbon along the beginning of the glycolate pathway was substantial. A large amount of ^{14}C flowed into active pools of glycolate and glycine and the labeling of those materials increased and reached or approached saturation (Figs. 45, 46). Tracer carbon flow from 3-PGA to glycerate and serine was also substantial (Figs. 30, 33). There may have been some accumulation of serine due to carbon flow from glycolate as well. Serine and glycerate labeling increased and both those substances reached saturation and had high steady state active pool sizes (Figs. 30, 33). When the level of $^{14}CO_2$ was increased to saturating (0.20-0.22%) the flow of tracer carbon into glycolate production, and along the glycolate pathway was apparently inhibited. This resulted in the observed decrease in the size of the photosynthetically active pools of glycolate and glycine (Figs. 45, 46). To the extent to which photorespiratory glycolate metabolism (including any glycolate to serine flow) was inhibited this also contributed to the increased photosynthetic

rates observed. Serine and glycerate labeling, however, did not decrease as would be expected if they were synthesized predominantly by the glycolate pathway. Serine and glycerate pool size instead remained constant, or increased under saturating $^{14}CO_2$ reflecting the pool size of PGA in each experiment (Figs. 30, 33). ^{14}C flow from PGA to glycerate and then serine was not inhibited by the $^{14}CO_2$ concentration increase. With a return to half saturating $^{14}CO_2$ in experiment B, glycolate production and carbon flow along the glycolate pathway increased and the active pool sizes of glycine and glycolate also increased reaching new higher steady state concentrations (Fig. 46). Some serine formation as a result of carbon flow from glycolate may have begun again. Glycerate and serine pool sizes, however, decreased reflecting predominantly the change in PGA pool size (Fig. 33), and not carbon flow along the glycolate pathway.

Evidence has been reported for formation of glycerate directly from 3-PGA in tobacco, soybean, coleus, peppermint and barley (Rabson et al. 1962, Hess and Tolbert 1966). Serine in those experiments, however, appeared to be synthesized predominantly from glycolate. The enzyme 3-phosphoglycerate phosphatase was found to be present in alfalfa (Randall et al. 1971). There has been some published evidence indicating serine production from 3-PGA in algae (Hess and Tolbert 1967, Bruin et al. 1970), the C_4 plant corn (Rabson et al. 1962), soybean leaves (Vernon and Aronoff 1950), tobacco leaves in the presence of glycolate metabolism inhibitor (Hess and Tolbert 1966), and in bean leaf discs (Vill and Parnik 1974, Voskresenskaya et al. 1970).

My observation of lessened glycolate production and repressed carbon flow along the glycolate pathway to glycine under saturating $^{14}CO_2$ conditions is in accord with much previous evidence. Carbon

flow along the glycolate pathway depends on glycolate synthesis, and contributes to photorespiratory CO_2 release. Both glycolate metabolism and photorespiration appear to generally be repressed by very high levels of CO_2 (Zelitch 1971 and 1975a, Chollet and Ogren 1975, Jackson and Volk 1970). For example, Whittingham and Pritchard (1963) reported that glycolate excretion during photosynthesis by Chlorella pyrenoidosa decreased as the CO_2 level was increased above 0.1%. Wilson and Calvin (1955), working with Scenedesmus, found increased labeled glycolate when the $^{14}CO_2$ level was changed from 1% to 0.03%. Robinson and Gibbs (1974) found that photosynthetic glycolate formation by isolated chloroplasts decreased when bicarbonate concentration was increased. Glycolate synthesis in tobacco tissue was inhibited by 0.5% CO_2 relative to air level CO_2 (Zelitch and Walker 1964). Egle and Fock (1967) found that at high levels of CO_2 (0.12%) the CO_2 post-illumination outburst did not occur in liverwort, or in attached bean and sunflower leaves. Inhibition of photosynthesis by oxygen (partially due to stimulation of photorespiration) was overcome by increased levels of CO_2 (Jackson and Volk 1970, Forrester et al. 1966a, Jolliffe and Tregunna 1968). Mahon et al. (1974) found that photorespiration in sunflower leaf discs was high and similar at 115 ppm CO_2 and 400 ppm CO_2, but was inhibited at 967 ppm CO_2.

Some authors have used label uptake into glycine and serine during fixed time leaf photosynthesis with $^{14}CO_2$ as an indication of the extent of photorespiration. Recent work includes that of Lee and Whittingham (1974) with tomato and Snyder and Tolbert (1974) with sugar beet and tobacco. That approach is of course based on the relationship between glycolate pathway metabolism and photorespiration. While Snyder and Tolbert (1974) equate photorespiration with glycolate

pathway metabolism, Lee and Whittingham (1974) seem to be aware that other metabolic pathways may contribute to photorespiratory CO_2 release. Given the nonglycolate path to serine suggested by Randall et al. (1971), which I have concluded actually operates in alfalfa, label uptake into serine in a given species of plant need not necessarily be reflective of glycolate pathway metabolism or of photorespiration. If serine is formed directly from 3-PGA during a given experiment then conclusions about glycolate pathway flow, and photorespiration, should not be based on serine label uptake data. To use serine data to draw conclusions about glycolate pathway flow one must first be certain it is being synthesized from glycolate and not PGA. Lee and Whittingham (1974) took this precaution and determined that serine was probably formed from glycine (and therefore glycolate) in their experiments. Hess and Tolbert (1966) showed earlier that serine probably arose from glycolate in tobacco. However, it is possible that the alternative origin of serine was overlooked as regards Snyder and Tolbert's (1974) work with sugar beet. Other experiments in which the possible alternative origin of serine may have been overlooked include that of Osmond and Bjorkman (1972) with Atriplex species and Mahon et al. (1974) with sunflower. At any rate, future work should take into account the possibility that serine can be formed by two paths. Furthermore, it would seem best to analyze ^{14}C uptake into glycolate, glycine, and serine separately when investigating glycolate pathway metabolism. This was not always done (Lee and Whittingham 1974, Snyder and Tolbert 1974) possibly due to glycolate volatility problems and glycine-serine separation problems. As was mentioned in Chapter I, the paper chromatography systems developed in this thesis eliminate these difficulties and make possible more

complete studies.

There may be some interspecial variation in the effect of elevated CO_2 on carbon flow along the glycolate pathway in C_3 plants. I have concluded that at saturating $^{14}CO_2$ (0.2%) glycolate formation and carbon flow along the glycolate pathway to glycine in alfalfa was strongly repressed relative to that at half saturating $^{14}CO_2$ (0.025%). Snyder and Tolbert (1974) have interpreted their data on the absolute amount of glycine+serine labeling during fixed term photosynthesis in tobacco as indicating that glycolate pathway flow was not repressed when the CO_2 concentration was increased from 0.03% to as high as 1.03%. Their sugar beet data, however, (leaving aside the origin of serine) showed a clear decrease in the absolute amount of glycine+serine labeling as CO_2 is increased above 0.03% and if interpreted on the same basis as their tobacco data indicates repression of glycolate metabolism at elevated CO_2. Lee and Whittingham (1974) concluded glycolate pathway metabolism was repressed in tomato at elevated CO_2.

As in Chapter I, it can be noted that conditions that repress photorespiration and therefore glycolate biosynthesis should decrease flow along the glycolate pathway from glycolate to glycerate. This could permit alternative flow from 3-PGA to glycerate and serine to predominate. The relatively low temperatures at which these alfalfa experiments were conducted (16 C - 18 C) may have repressed photorespiration at both half saturating and saturating $^{14}CO_2$ (Zelitch 1971, Jackson and Volk 1970) and favored predominant serine and glycerate synthesis from 3-PGA by repressing glycolate metabolism. I, however, do not believe this to have been a significant factor. Firstly, the gas exchange measurements indicated photorespiration, and therefore glycolate metabolism, was probably still substantial at 17 C. Secondly,

the quantities of label found in glycolate and glycine after several minutes of photosynthesis with half saturating $^{14}CO_2$ were substantial and comparable to those reported at near air level $^{14}CO_2$ in other C_3 species (Rabson et al. 1962, Osmond and Bjorkman 1972, Lee and Whittingham 1974). Carbon flow along the start of the glycolate pathway in alfalfa therefore appeared to have been substantial at half saturating $^{14}CO_2$. Furthermore, when the $^{14}CO_2$ level was raised and glycolate metabolite pool size, and flow along the glycolate pathway, apparently decreased (Figs. 45, 46), serine and glycerate pool sizes either remained constant or actually increased (Figs. 30, 33). All these observations, and also the substantial level of label accumulating in serine and glycerate throughout experiments A and B (Figs. 30, 33; Chapter II Results) provide strong evidence that the path 3-PGA→ glycerate↠serine is of major metabolic importance in alfalfa, and is not a minor residual path.

E. Conclusions and Summary

1. The photosynthetic rate of alfalfa leaflets was found to be strongly dependent on both oxygen and carbon dioxide concentration. Curves relating those concentrations to photosynthetic rate were obtained.

2. The CO_2 compensation point, dark respiration rate, and estimated photorespiratory rate of alfalfa leaflets were obtained.

3. The gas exchange parameter data were in accord with alfalfa being a C_3 plant. That data supported the occurrence of photo-respiration in alfalfa.

4. The gas exchange data were in accord with the idea that alfalfa crop productivity could be enhanced through atmospheric manipulation.

5. The apparatus recently developed to study steady state photosynthesis by whole alfalfa leaflets with $^{14}CO_2$ was applied to the investigation of a perturbed steady state situation. In particular the situation in which the $^{14}CO_2$ concentration available to the alfalfa leaflets was increased from half saturating to saturating was investigated. In one experiment the $^{14}CO_2$ concentration was also returned to half saturating concentration.

a) Increasing the $^{14}CO_2$ concentration available to whole alfalfa leaflets from half saturating to saturating concentration resulted in an approximately doubled rate of photosynthesis.

b) The photosynthetic rate increase at saturating $^{14}CO_2$ was accounted for by increased labeling of both soluble and insoluble products.

c) A large increase in carbon flow through the RPP cycle occurred at saturating $^{14}CO_2$ with only a small absolute increase in the amounts of recently fixed carbon accumulating in RPP cycle metabolites. The photosynthetic cycle intake and carbon flow take offs were well balanced. The data were suggestive of a regulatory interaction between $^{14}CO_2$ transport into the chloroplast and ^{14}C-metabolite transport out of it.

d) Tracer carbon flow to the TCA cycle intermediates, and the amino acids derived from those intermediates, participated in the overall increased rate of $^{14}CO_2$ incorporation at elevated $^{14}CO_2$. Particularly noticeable were an alanine pool size increase, and pool size and labeling rate increases observed for citrate, aspartate, and glutamate at elevated $^{14}CO_2$. Carbon flow appeared to be along the paths shown in Fig. 23, with elevated $^{14}CO_2$ resulting in generally increased tracer carbon flow from the RPP cycle to the TCA cycle

metabolites, amino acid, and probably protein synthesis. There was some evidence for activation of pyruvate kinase at elevated $^{14}CO_2$.

e) Sucrose was the major soluble labeled product formed. Pool sizes of UDPG and F6P increased at elevated $^{14}CO_2$ and brought about increased rates of sucrose synthesis. Sucrose labeling appeared to account for a slightly greater proportion of the overall fixation rate at saturating $^{14}CO_2$ vs. half saturating $^{14}CO_2$.

f) Glycolate production and the flow of recently fixed carbon along the glycolate pathway was apparently inhibited at elevated $^{14}CO_2$. Glycine appeared to be synthesized from glycolate by the glycolate pathway. Glycolate and glycine photosynthetically active pools decreased in size at elevated $^{14}CO_2$. Repression of glycolate metabolism and any associated photorespiration at elevated $^{14}CO_2$ probably contributed to the observed photosynthetic rate increase. However, much of that increase may be due to increased rates of photosynthesis and not repression of photorespiration.

g) Glycerate and serine were concluded to be predominantly synthesized directly from 3-PGA and not from glycolate by the glycolate pathway. At elevated $^{14}CO_2$ their pool sizes remained constant in one experiment, and increased in a second in both cases reflecting the pool size of PGA. Their pool sizes did not decrease at elevated $^{14}CO_2$ as did those of glycolate and glycine. Carbon flow from 3-PGA to glycerate and serine appeared to be a major metabolic path in alfalfa, and was apparently not inhibited by elevated $^{14}CO_2$.

h) Pool size and labeling rate changes which occurred on increasing the $^{14}CO_2$ concentration were generally reversible. Pool sizes and labeling rates responded as expected to a return to lower $^{14}CO_2$ concentration.

6. In work with species other than alfalfa, greater recognition must be given the idea that glycerate and serine may be synthesized from 3-PGA directly and not from glycolate. Glycerate and serine data must be interpreted with caution. Combined glycine and serine labeling data should generally not be used as pool sizes and production of those amino acids may respond oppositely to some biochemical perturbations.

7. A large amount of data has been accumulated during these investigations on photosynthesis in alfalfa. The steady state exposure apparatus developed has been shown to be capable of being used successfully to study perturbed steady state photosynthesis with $^{14}CO_2$ by whole leaflets. It is the author's intention to make further use of the unique steady state apparatus developed, and continue investigation of alfalfa photosynthetic metabolism using the techniques of perturbed steady state photosynthesis.

REFERENCES

Atkins, C. A., and Canvin, D. T. (1971) Can. J. Bot. 49: 1225-1233.

Bassham, J. A. (1964) Annu. Rev. Plant Physiol. 15: 101-120.

(1971) Science 172: 526-534.

(1973) In "Symposia of the Society for Experimental Biology, Rate Control of Biological Processes", 27: 461-483. University Press, Cambridge.

Bassham, J. A., and Calvin, M. (1957) "The Path of Carbon in Photosynthesis." Prentice-Hall, Englewood Cliffs, N.J.

Bassham, J. A., and Kirk, M. (1960) Biochim. Biophys. Acta 43: 447-464.

(1962) Biochem. Biophys. Res. Commun. 9: 376-380.

(1964) Biochim. Biophys. Acta 90: 553-562.

(1973) Plant Physiol. 52: 407-411.

Bassham, J. A., and Krause, G. H. (1969) Biochim. Biophys. Acta 189: 207-221.

Bassham, J. A., Morawiecka, B., and Kirk, M. (1964) Biochim. Biophys. Acta 90: 542-552.

Bishop, P. M., and Whittingham, C. P. (1968) Photosynthetica 2: 31-38.

Bjorkman, O., and Berry, J. (1973) Sci. Amer. 229: 80-93.

Black, C. C. (1973) Annu. Rev. Plant Physiol. 24: 253-286.

Bowes, G., Ogren, W. L., and Hageman, R. H. (1971) Biochem. Biophys. Res. Commun. 45: 716-722.

Brown, R. H., Pearce, R. B., Wolf, D. D., and Blaser, R. E. (1972) In "Alfalfa Science and Technology" (C. H. Hanson, ed.), pp. 143-166. Am. Soc. Agronomy, Inc., Madison, Wisconsin.

Bruin, W. J., Nelson, E. B., and Tolbert, N. E. (1970) Plant Physiol. 46: 386-391.

Bruinsma, J. (1963) Photochem. Photobiol. 2: 241-249.

Calvin, M., and Massini, P. (1952) Experientia 8: 445-457.

Canvin, D. T., and Fock, H. (1972) In "Methods in Enzymology" 24 B (A. San Pietro, ed.), pp. 246-260. Academic Press, N. Y. and London.

Chapman, E. A., and Graham, D. (1974) Plant Physiol. 53: 879-885.

Chollet, R., and Ogren, W. L. (1975) Bot. Rev. 41: 137-179.

Consden, R., Gordon, A. H., and Martin, A. J. P. (1944) Biochem. J. 38: 224-232.

Decker, J. P. (1955) Plant Physiol. 30: 82-84.

(1957) J. Sol. Energy Sci. Eng. 1: 30-33.

Delaney, R. H., Dobrenz, A. K., and Poole, H. T. (1974) Crop Sci. 14: 58-61.

Edwards, R. H., Miller, R. E., DeFremery, D., Knuckles, B. E., Bickoff, E. M., and Kohler, G. O. (1975) J. Agric. Food Chem. 23: 620-626.

Egle, K., and Fock, H. (1967) In "Biochemistry of Chloroplasts" (T. W. Goodwin, ed.), Vol. 2, pp. 79-87. Academic Press, N. Y.

Fink, K., Cline, R. E., and Fink, R. M. (1963) Anal. Chem. 35: 389-398.

Fock, H., Mahon, J. D., Canvin, D. T., and Grant, B. R. (1974) In "Mechanisms of Plant Growth" (R. Bieleski, A. R. Ferguson, M. M. Creswell, eds.), pp. 235-242, Bulletin 12, The Royal Society of New Zealand, Wellington.

Ford, M. A., and Thorne, G. N. (1967) Annals of Botany, N. S. 31: 629-644.

Forrester, M. L., Krotkov, G., and Nelson, C. D. (1966a) Plant Physiol. 41: 442-427.

(1966b) Plant Physiol. 41: 428-431.

Foutz, A. L., Wilhelm, W. W., and Dobrenz, A. K. (1976) Agron. J. To be published.

Fowden, L. (1965) In "Biosynthetic Pathways in Higher Plants" (J. B. Pridham and T. Swain, eds.), pp. 73-99. Academic Press, N. Y. and London.

Gaastra, P. (1959) Meded. Landbouwhogesch. Wageningen 59: 1-68.

(1962) Neth. J. Agric. Sci. 10: 311-324.

Gale, J., Kaplan, A., and Tako, T. (1975) J. Exp. Bot. 26: 702-704.

Hardy, R. W. F., and Havelka, U. D. (1975) Science 188: 633-643.

Hatch, M. D. (1971) In "Photosynthesis and Photorespiration" (M. D. Hatch, C. B. Osmond, R. O. Slayter, eds.), pp. 139-152. Wiley-Interscience, New York.

Heber, U. (1974) Annu. Rev. Plant Physiol. 25: 393-421.

Heber, U., and Willenbrink, J. (1964) Biochim. Biophys. Acta 82: 313-324.

Hess, J. L., and Tolbert, N. E. (1966) J. Biol. Chem. 241: 5705-5711.

(1967) Plant Physiol. 42: 371-379.

Hew, C., Krotkov, G., and Canvin, D. T. (1969) Plant Physiol. 44: 662-670.

Hill, R. (1937) Nature 139: 881-882.

Hill, R., and Scarisbrick, R. (1940a) Proc. R. Soc. London B129: 238-255.

(1940b) Nature 146: 61-62.

Huffaker, R., Cox, E. L., Kleinkopf, G. E., and Stanford, E. H. (1970) Physiol. Plant. 23: 404-411.

Jackson, W. A., and Volk, R. J. (1970) Annu. Rev. Plant Physiol. 21: 385-432.

Jacoby, B., and Laties, G. G. (1971) Plant Physiol. 47: 525-531.

Jensen, R. G., and Bassham, J. A. (1966) Proc. Natl. Acad. Sci. USA 56: 1095-1101.

Joliffe, P. A., and Tregunna, E. B. (1968) Plant Physiol. 43: 902-906.

Kanazawa, T., Kirk, M., and Bassham, J. A. (1970) Biochim. Biophys. Acta 205: 401-408.

Krenzer, E. G., Moss, D. N., and Crookston, R. K. (1975) Plant Physiol. 56: 194-206.

Laetsch, W. M. (1974) Annu. Rev. Plant Physiol. 25: 27-52.

Laing, W. A., Ogren, W. L., and Hageman, R. H. (1974) Plant Physiol. 54: 678-685.

Lee, R. B., and Whittingham, C. P. (1974) J. Exp. Bot. 25: 277-287.

Ludlow, M. M., and Jarvis, P. G. (1971) In "Plant Photosynthetic Production Manual of Methods" (see Setlik, I., and Sestak, Z., 1971), pp. 294-315.

Ludwig, L. J., and Canvin, D. T. (1971) Plant Physiol. 48: 712-719.

McNaughton, S. J., and Fullem, L. W. (1970) Plant Physiol. 45: 703-707.

MacDonald, I. R. (1975) Plant Physiol. 56: 109-112.

Macnicol, P. K. (1976) Plant Physiol. 57: 80-84.

Madsen, E. (1974) Physiol. Plant. 32: 10-13.

Mahon, J. D., Fock, H., and Canvin, D. T. (1974) Planta (Berl.) 120: 245-254.

Moses, V., and Lonberg-Holm, K. K. (1963) Anal. Biochem. 5: 11-27.

Moss, D. N. (1962) Nature 193: 587.

(1964) Crop Sci. 4: 131-136.

Norris, L., Norris, R. E., and Calvin, M. (1955) J. Exp. Bot. 6: 64-74.

Osmond, C. B., and Bjorkman, O. (1972) Carnegie Inst. Washington Yearbook 71: 141-148.

Parker, H. I. (1971) Ph.D. Thesis, University of California, Berkeley.

Pearce, R. B., Brown, R. H., and Blaser, R. E. (1968) Crop Sci. 8: 677-680.

Pearce, R. B., Carlson, G. E., Barnes, D. K., Hart, R. H., and Hanson, C. H. (1969) Crop Sci. 9: 423-426.

Pedersen, T. A., Kirk, M., and Bassham, J. A. (1966a) Biochim. Biophys. Acta 112: 189-203.

(1966b) Physiol. Plant. 19: 219-231.

Pirie, N. W. (1966) Science 152: 1701-1705.

Rabinowitch, E. I. (1945) "Photosynthesis and Related Processes." Vol. I, Interscience, N.Y., N.Y.

Rabson, R., Tolbert, N. E., and Kearney, P. C. (1962) Arch. Biochem. Biophys. 98: 154-163.

Randall, D. D., Tolbert, N. E., and Gremel, D. (1971) Plant Physiol. 48: 480-487.

Robinson, J. M., and Gibbs, M. (1974) Plant Physiol. 53: 790-797.

Ruben, S., Hassid, W. Z., and Kamen, M. D. (1939) J. Am. Chem. Soc. 61: 661-663.

Ruben, S., and Kamen, M. D. (1940) Proc. Natl. Acad. Sci. USA 26: 418-426.

(1941) J. Appl. Phys. 12: 321-322.

Sawada, S., and Miyachi, S. (1974) Plant Cell Physiol. 15: 225-238.

Setlik, I., and Sestak, Z. (1971) In "Plant Photosynthetic Production Manual of Methods" (Z. Sestak, J. Catsky, P. G. Jarvis, eds.), pp. 316-342. Dr. W. Junk N. V. Publishers, The Hague.

Shomer-Ilan, A., Beer, S., and Waisel, Y. (1975) Plant Physiol. 56: 676-679.

Smith, D. C., Bassham, J. A., and Kirk, M. (1961) Biochim. Biophys. Acta 48: 299-313.

Snyder, F. W., and Tolbert, N. E. (1974) Plant Physiol. 53: 514-515.

Stahmann, M. A. (1968) Econ. Bot. 22: 73-79.

Strain, H. H., and Svec, W. C. (1966) In "The Chlorophylls" (L. P. Vernon and G. R. Seely, eds.), pp. 21-66. Academic Press, N. Y. and London.

Tamas, I. A., and Bidwell, R. G. S. (1970) Can. J. Bot. 48: 1259-1263.

Ting, I. P., and Osmond, C. B. (1973a) Plant Physiol. 51: 439-447.

(1973b) Plant Physiol. 51: 448-453.

Tolbert, N. E. (1971) In "Photosynthesis and Photorespiration." (see Hatch, M. D. 1971), pp. 458-471.

(1973a) In "Current Topics in Cellular Regulation." (B. L. Horecker and E. R. Stadtman, eds.), pp. 21-50. Academic Press, New York and London.

(1973b) In "Symposia of the Society for Experimental Biology 27, Rate Control of Biological Processes", pp. 215-239. University Press, Cambridge.

Turner, W. B., and Bidwell, R. G. S. (1965) Plant Physiol. 40: 446-451.

van Niel, C. B. (1931) Arch. Mikrobiol. 3: 1-112.

Vernon, L. P., and Aronoff, S. (1950) Arch. Biochem. 29: 179-186.

Vill, J., and Parnik, T. (1974) Photosynthetica 8: 208-215.

Voskresenskaya, N. P., Wiil, Y. A., Grishina, G. S., and Parnik, T. R. (1970) Photosynthetica 4: 1-8.

Whittingham, C. P., and Pritchard, G. G. (1963) Proc. R. Soc. London B 157: 366-380.

Wilson, A. T., and Calvin, M. (1955) J. Am. Chem. Soc. 77: 5948-5957.

Wittwer, S. H. (1974) BioScience 24: 216-224.

Wittwer, S. H., and Robb, W. (1964) Econ. Bot. 18: 34-56.

Wolf, D. D., and Blaser, R. E. (1972) Crop Sci. 12: 23-26.

Zelitch, I. (1965) J. Biol. Chem. 240: 1869-1876.

(1971) "Photosynthesis, Photorespiration, and Plant Productivity." Academic Press, New York.

(1973) Proc. Natl. Acad. Sci. USA 70: 579-584.

(1975a) Science 188: 626-633.

(1975b) Annu. Rev. Biochem. 44: 123-145.

Zelitch, I., and Walker, D. A. (1964) Plant Physiol. 39: 856-862.

TECHNICAL INFORMATION DIVISION
LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
BERKELEY, CALIFORNIA 94720