EXPERIMENTAL PLANT PHYSIOLOGY

Anthony San Pietro

EXPERIMENTAL PLANT PHYSIOLOGY

Edited by

Anthony San Pietro

Department of Plant Sciences, Indiana University,
Bloomington, Indiana

WITH 26 CONTRIBUTORS

with 30 *illustrations*

The C. V. Mos
SAINT LOUIS 1974

Printed in the United States of America

Distributed in Great Britain by Henry Kimpton, London

Library of Congress Cataloging in Publication Data

San Pietro, Anthony Gordan, 1922-
Experimental plant physiology.

1. Plant physiology—Experiments I Title
[DNLM: 1. Plants—Physiology. QK711 2 S288e 1974]
QK714.4.S26 581.1'07'24 73-20407
ISBN 0-8016-4307-4

TS/M/M 9 8 7 6 5 4 3 2 1

Contributors

Alva A. App

Department of Cell Physiology and Virology, Boyce Thompson Institute for Plant Research, Inc., Yonkers, New York

Donald J. Armstrong

Department of Botany, University of Wisconsin, Madison, Wisconsin

Mordhay Avron

Biochemistry Department, Weizmann Institute of Science, Rehovot, Israel

James A. Bassham

Lawrence Radiation Laboratory, University of California, Berkeley, California

Leonard Beevers

Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma

Lawrence Bogorad

Department of Biology, Harvard University, Cambridge, Massachusetts

Walter D. Bonner, Jr.

Johnson Research Foundation, University of Pennsylvania, Philadelphia, Pennsylvania

R. William Breidenbach

Department of Agronomy and Range Science, University of California, Davis, California

Arthur W. Galston

Department of Biology, Yale University,
New Haven, Connecticut

Donald R. Geiger

Department of Biology, University of Dayton,
Dayton, Ohio

Martin Gibbs

Department of Biology, Brandeis University,
Waltham, Massachusetts

Andre T. Jagendorf

Section of Genetics Development and Physiology, Cornell University,
Ithaca, New York

Merrill R. Kaufmann

Department of Plant Sciences, University of California,
Riverside, California

George G. Laties

Biology Department, University of California,
Los Angeles, California

A. Carl Leopold

Department of Horticulture, Purdue University,
Lafayette, Indiana

Jacob Levitt

Department of Biological Sciences, University of Missouri,
Columbia, Missouri

C. O. Manahan

Department of Cell Physiology and Virology, Boyce Thompson Institute
for Plant Research, Inc., Yonkers, New York

Stephan Miller

Department of Biology, Harvard University,
Cambridge, Massachusetts

B. W. Poovaiah

Department of Horticulture, Purdue University,
Lafayette, Indiana

J. Michael Robinson

Department of Biology, Brandeis University,
Waltham, Massachusetts

Ruth L. Satter

Department of Biology, Yale University,
New Haven, Connecticut

Folke Skoog

Department of Botany, University of Wisconsin,
Madison, Wisconsin

Joseph E. Varner

Department of Biology, Washington University,
St. Louis, Missouri

Hubert B. Vickery

Department of Biochemistry, The Connecticut Agricultural
Experiment Station, New Haven, Connecticut

Jan A. D. Zeevaart

Plant Research Laboratory, Michigan State University,
East Lansing, Michigan

Israel Zelitch

Department of Biochemistry, The Connecticut Agricultural
Experiment Station, New Haven, Connecticut

Preface

The laboratory work associated with many biology courses serves merely as an extension of the lecture presentation. As such it is accepted hesitatingly, certainly not enthusiastically, by many students who view the laboratory as little more than a place to "put in time." This is most unfortunate since the laboratory, properly conceived, can be a powerful teaching and investigative vehicle. The very important potential role of the laboratory was convincingly stated by Dr. Ruth C. Von Blum in her recent article in the *AIBS Education Review:*

> One of the primary objectives of any course in science is to educate students to carry out scientific work at some level of competence. This involves the development of proficiency in three fundamental activities: (1) careful observation, (2) the use of scientific instruments and techniques, and (3) the process of scientific investigation. The laboratory can be specifically designed to reach these objectives, but laboratories in most biology courses are viewed primarily as vehicles for observing material presented in lecture. The training aspect is usually treated peripherally and casually. The investigatory component is most often neglected.*

The present volume was conceived initially to alleviate the very weakness noted above and to bring the laboratory into proper educational focus. Toward this end, each chapter has been written by an acknowledged leader in his field of research; further, most are university faculty members and teach at the undergraduate level. The wide variety of experimental approaches described is truly representative of the current research efforts in the various areas and is in everyday use in the authors' own laboratories. The following few examples may be cited: two-dimensional chromatography, which was instrumental in elucidating the path of carbon in photosynthesis; measurement of vectorial proton translocation, as derived from the Mitchell chemiosmotic hypothesis for the mechanism of ATP formation;

*Vol. 2, No. 2, April 1973.

determination of the relationship between in vitro protein synthesis and synthetic messenger RNA, an experiment similar in principle to that used to determine the genetic code; and the use of in vitro culture of tobacco callus tissue as a sensitive and specific bioassay for cytokinins.

In the opening section of each chapter, the student is provided with an assessment of the problem and the current status of the research efforts in that area of investigation. Thereafter follows a detailed description of the experimental procedures, including collection, display, and calculation of the data, plus questions to be considered and optional additional experiments that can be pursued if time permits. Although this volume contains more experiments than can be accommodated in the normal one-semester course, this was done deliberately to provide sufficient diversity and flexibility of experimental approaches for students and instructors with varied interests and facilities. This volume will be of use to undergraduates as well as beginning graduate students and investigators in allied fields.

I deem it a distinct honor and pleasure to be associated with this endeavor. Any success this volume may achieve in elevating the laboratory to its proper stature in science education will be due solely to the efforts of the individual authors. Their willingness to cooperate in this venture is gratefully acknowledged.

Anthony San Pietro

Contents

1

^{14}C Labeling and two-dimensional paper chromatography of plant metabolites

James A. Bassham

ADVANTAGES OF PAPER CHROMATOGRAPHIC ANALYSIS AND RADIOAUTOGRAPHY FOR STUDIES IN INTERMEDIARY METABOLISM

A great many biochemical reactions occur simultaneously within a living cell. Many classical methods of study of intermediary metabolism are able to examine only a few of the substances involved in these reactions in any single experiment. In contrast, analysis by two-dimensional paper chromatography of labeled compounds formed from labeled substrates permits an examination of numerous different compounds simultaneously. Kinetic studies using this method provide a dynamic picture of the flow of material through the metabolic pathways.

Metabolic intermediates in green plant cells are readily labeled by the introduction of radioactive tracers such as carbon 14, phosphorus 32, or both. Carbon 14 may be administered as $^{14}CO_2$ to leaves of plants or as $H^{14}CO_3^-$ in solution to aquatic plants. Phosphorus 32 can be administered as inorganic phosphate in solution directly in the medium of aquatic plants or to the roots or by injection into the veins of leaves of plants. Green plants very quickly incorporate the radioactive tracer into a large number of metabolic intermediates. In photosynthesizing cells, these metabolites include 3-phosphoglycerate, a number of sugar phosphates and sugar diphosphates, amino acids, carboxylic acids, and other compounds of small molecular weight. Some radioactivity is also incorporated into macromolecules in relatively short periods of time.

Two-dimensional paper chromatography is an extremely useful method of separating a large number of diverse intermediary metabolites. Other chromatographic methods, such as thin-layer chromatography, column chromatography with ion exchange resins, vapor phase chromatography, and so on, are also suitable for separations of some of these classes of compounds formed in green plant cells. However, for a one-step separation of many labeled compounds, two-dimensional paper chromatography is probably the most useful method. First, two-dimensional paper chromatography provides a high degree of resolution of a broad spectrum of intermediary metabolites. Second, the shape and fine structure of the areas of paper covered by individual compounds are highly distinctive, permitting a kind of fingerprinting and nearly absolute identification when radioactive compounds are found to coincide with carrier unlabeled compounds. Third, when radioactive compounds are being analyzed, extremely small quantities may be detected by paper chromatography followed by radioautography with medical x-ray film.

The use of paper chromatography in amino acid separation by Consden (1) in 1944

led to the application of this technique by Calvin and co-workers (2) to the analysis of products of photosynthesis with $^{14}CO_2$ in green plants. This method, described more fully by Benson and others (3), was of key importance in the elucidation of the path of carbon fixation in photosynthesis via the reductive pentose phosphate cycle (4, 5). Since that time the method has been used extensively to study not only the pathways of carbon fixation in photosynthetic plants but also the mechanisms of the regulation of metabolism in green plants (6, 7). Over the years, improvements have been made in the method that have resulted in chromatograms superior in resolution to those obtained in the early work (6).

PRINCIPLES OF PAPER CHROMATOGRAPHY

As with many other types of chromatography, paper chromatography depends upon the partition of the compounds between two different phases. In the case of paper chromatography, these are liquid phases. One liquid phase is stationary and is absorbed on the cellulose fibers in the paper. In general, the stationary phase is predominantly aqueous but contains organic solvents mixed into it. The mobile phase is predominantly organic but contains water dissolved in it. If α represents the distribution coefficient of a particular solvent between the two phases, the relative rate of movement of that solute is given by the equation $R_F = A_L/(A_L + \alpha A_S)$. A_L is the fraction of the cross-sectional area of the chromatogram occupied by the mobile phase, and A_S is the fraction of the cross-sectional area occupied by the stationary phase. R_F is defined as the distance from the origin to the position of the solute, divided by the distance from the origin to the solvent front.

Benson and others tested this theory (3) with a variety of carboxylic acids and sugars from green plant cells by distributing these compounds between an organic and an aqueous phase in separatory funnels and by comparing the calculated R_F with the measured R_F. Fairly good agreement with the theory was obtained. However, it should be noted that with some compounds additional effects were seen that might be attributed to absorption of compound by the cellulose fibers.

With this theory in mind, the investigator finds that the selection of suitable chromatographic solvents becomes a problem of finding solvents in which the majority of the solutes to be separated will be distributed to some extent between the two phases but with a variety of distribution coefficients, depending upon the physical properties of the particular compound. For two-dimensional paper chromatography, the two chromatographic solvents should have not too similar properties, since if they were exactly the same, a diagonal row of compounds would be produced by equivalent resolution in the two directions. One way to achieve two different types of resolution for the two dimensions of chromatography is to make one solvent acidic and the other basic. However, most of the compounds of interest in intermediary metabolism of photosynthetic green cells are either neutral or acidic—that is, they are either neutral amino acids, acidic amino acids, carboxylic acids, sugars or sugar phosphates, or sugar nucleotide phosphates. For such compounds, basic solvents are generally not very effective, since many substances tend to be held back and run together. Therefore, it has been found that most products of photosynthesis and intermediary metabolism in green cells are best separated by two acidic solvents with rather different solvent properties.* The first solvent used is

* An exception is glycolic acid, which is volatile and thus is partly lost from papers developed in acidic solvents. A basic solvent (for example, one containing added NH_4OH) can be used for the separation of glycolate from other metabolites. When the chromatogram is dried, the ammonium salt of glycolic acid is not lost.

phenol with a nearly saturating amount of water. Sufficiently pure phenol can now be purchased in the form of liquified phenol, which already contains some water. To this is added enough water to raise it to the desired condition of just under saturation. Since such phenol commonly is acidic as purchased because of impurities, it is desirable to maintain a constant acidity by adding additional acid to reduce the variability from one lot of chromatographic solvent to another. This is achieved by adding glacial acetic acid. A small amount of 1 M ethylenediaminetetraacetic acid (EDTA) is also added, which forms a complex with divalent metallic ions and removes them from the paper. This in turn greatly improves the chromatography of sugar phosphates and other phosphorylated compounds, which would tend to react with the divalent ions in the paper.

A second solvent commonly used is a mixture of butanol, propionic acid, and water. The solvent properties of propionic acid are not greatly different from those of acetic acid, but the solvent properties of butanol, which constitutes a major part of the second solvent, are very different from the solvent properties of phenol, which constitutes the major part of the first solvent. Thus, a different kind of separation is achieved in the two directions, even though both solvents are acidic.

Two-dimensional paper chromatograms are prepared in research laboratories by applying a suspension or solution of the biochemical mixture to be separated to a small area near one corner of a large sheet of suitable chromatographic filter paper. This point of application is called the origin. One edge of the paper next to the origin is folded and placed in a dry chromatographic trough. A second paper is usually placed in the same trough so that the folds of the two papers overlap and the papers hang down from the trough on opposite sides. Usually each paper hangs over an antisiphoning rod that is horizontal and slightly above and separated from the top edge of the trough by a small space (about 5 mm) so that siphoning of solvent between paper and trough edge does not occur. A bar weight is then placed on top of the folds of the papers.

Papers and trough are then placed in a chromatographic cabinet that is vapor tight, and the chromatographic solvent is added to the trough. The solvent travels by capillarity through the paper, moving at a uniform rate down the paper and across the origin. As it passes the origin, it dissolves the compounds that were applied there and carries them along with the solvent at a rate less than the rate of travel of the solvent front. In some cases the solvent front is allowed to reach the far edge of the paper, whereas in other cases it is allowed to drip from the far edge of the paper in descending chromatography. This depends upon how rapidly the compounds to be separated are moving—that is, their R_f values. When the separation is deemed complete, the experimenter dries the paper either by removing the paper from the box and hanging it in a hood or, preferably if the equipment is available, by applying a suction to the box to draw a stream of air through the box into an exhaust system. The time for development of the paper in the first solvent may vary from 6 hr to 48 hr or more, depending upon the solvents used and the R_fs of the compounds to be separated.

After drying, the paper is rotated 90°, and the other edge of the paper is placed in another trough for chromatography in the second dimension. The weight is again added to the paper fold, the trough and papers are placed in the box, the solvent is added, and development proceeds as just described. When this second development is completed, the paper is again dried, at which point it is ready for radioautography.

The paper is folded around a large sheet of medical x-ray film, and the packet is

placed in a light-tight cassette for a few days to allow the radioactive emanations from the labeled compounds to expose the x-ray film. The film is then developed, and dark spots appear wherever radioactive compounds were in contact with the film.

EXPOSURE OF PLANT TISSUE TO RADIOACTIVITY

For studies of metabolites of photosynthesis, leaves, suspensions of unicellular algae, or suspensions of isolated chloroplasts may be used. For either suspensions of algae or of chloroplasts, the biologic material is suspended in a suitable buffer. A solution of ^{14}C-labeled bicarbonate or ^{32}P-labeled phosphate or both is added. The suspension is illuminated for a minute or so with gentle swirling, after which the biologic material is killed by addition of methanol to a final concentration of 80% at room temperature. The methanol quickly dissolves the lipid membranes of the cell and denatures the protein enzymes, thereby stopping the biochemical reactions. For kinetic measurements, samples of the algae or chloroplasts are removed from time to time and separately killed.

For experiments with leaves, the leaves of a small plant are placed in a chamber with transparent walls or windows through which illumination is provided. Gaseous $^{14}CO_2$ is added or is generated within the chamber by addition of acid to $Ba^{14}CO_3$. After a short period of photosynthesis, the leaves are removed from the chamber and killed. An aliquot sample of the entire suspension of dead biologic material is then applied to the origin of a paper chromatogram. An air current from a hair dryer may be used to facilitate drying, leaving the biologic material on the origin. If a hair dryer is used, the temperature should be set fairly low so that those compounds that are chemically labile are not destroyed. Alternatively, a stream of air or nitrogen may be used.

LABORATORY EXPERIMENTS

Several variations of the techniques used in research studies are described since some student laboratories may not be equipped with the large chromatographic boxes required for paper chromatograms measuring approximately 46 cm × 57 cm. However, the principles may be adequately tested with much smaller chromatograms, of the order of 25 cm × 25 cm. Also, experiments are described for the use of unicellular algae or leaves. Either of these biologic experiments may be combined with analysis by either the large or the small chromatograms.

Materials

Plants. Healthy leaves of land plants capable of high rates of photosynthesis and of such a texture as to be easily extractable with organic solvents are required. Among plants that have been successfully studied are spinach, soybean, pea, and alfalfa. Plants that exhibit the additional pyruvate-malate pathway of CO_2 fixation, such as maize or sugar cane, may also be used but are often more difficult to extract because of the fibrous nature of their leaves.

To allow each student to do an experiment, a single alfalfa leaf about 2 cm long (or other leaf of similar size) is used. Whatever leaves are chosen, they should be harvested, kept on ice, and used the same day as picked, if possible.

Among fresh-water aquatic plants, unicellular green algae, such as *Chlorella pyrenoidosa*, have been widely used in research and are very suitable. Marine algae present a serious chromatographic problem because of the high salt content in their growth medium.

Radiocarbon. For leaves and higher plants, $^{14}CO_2$ mixed with $^{12}CO_2$ is used. For algal and chloroplast suspensions, a solution of $NaH^{14}CO_3$ mixed with unlabeled bicarbonate is used. In either case, a specific radioactivity of at least 10 microcuries/μmole should be used so that the small amounts of materials on the paper chromatograms will contain enough ^{14}C for easy detection. For class use, one ml of a 0.05 M solution of $NaH^{14}CO_3$ and $NaH^{12}CO_3$ with a specific radioactivity of 20 microcuries/μmole is recommended. This solution, containing 1 millicurie of ^{14}C, is placed in a 2-ml serum bottle with a tight-fitting serum cap. For all experiments, each student may withdraw 20 μl (20 microcuries) with a 0.1-ml graduated microsyringe equipped with a 2½ inch, No. 20 gauge hypodermic needle. All ^{14}C-contaminated glassware and needles should be rinsed with dilute HCl in a fume hood.

Chromatographic solvents. The solvent for the first dimension contains 840 ml "liquified" phenol (Mallinckrodt, about 88% phenol and 12% water), 160 ml water, 10 ml glacial acetic acid, and 1 ml of 1.0 M EDTA (6).

The solvent for the second dimension is prepared by mixing together just prior to chromatography equal volumes of the following two mixtures (6): n-butanol:water (370:25 v/v) and propionic acid:water (180:220 v/v).

Chromatographic paper. For paper chromatography, a fine-grained, chromatographic-grade filter paper is required for best results. One paper often used in research work is Whatman No. 1. Care should be taken to ensure that the first solvent (phenol-water) is run with the grain of the paper. This is difficult to see in a fine-grained paper, but is usually specified in a chromatographic-grade paper. The grain of Whatman No. 1 is the long direction of the paper.

Formerly, the paper was often washed with a solution of oxalic acid or of EDTA to remove divalent cations, which could interfere with the movement of phosphate esters on the paper. This laborious paper washing is now eliminated by addition of the EDTA to the phenol solvent.

Radioactive ink. For exact alignment of x-ray films with paper chromatograms after development, it is desirable to label the corners of the chromatographic paper with radioactive ink. Thus, when the film is developed, the ink on the paper can be exactly matched with the corresponding exposed mark on the paper. In this way, all radioactive spots on the paper are precisely located with respect to dark spots on the film. The ink can be prepared by the addition of nonvolatile radioactive compounds (such as ^{14}C-labeled glucose) to ordinary black ink.

X-ray film. Single-coated, blue-sensitive medical x-ray film (such as Kodak SB-54) is used. Double-coated film could be used but would have twice the background (because of cosmic radiation), and only one side would be exposed by the weak beta rays from the ^{14}C.

Other materials. Among other materials required are methanol for killing the plants, liquid N_2 and dry ice if leaves are to be killed and extracted, microsyringes and needles, and various standard biochemical compounds, such as amino acids (alanine, aspartate, and glutamate), sugars, carboxylic acids, and so on, for identification purposes. Ninhydrin may be employed as a color reagent spray for amino acids.

Equipment

Chambers for plants or leaves. A chamber for an experiment with a whole small plant or with excised whole leaves must be transparent or have windows to provide for illumination, and it must have either inlet and outlet valves for introducing CO_2 or provision for generation of $^{14}CO_2$ within the chamber. The actual design will depend on the size and shape of the plants and materials available.

For a small leaf, such as a single alfalfa leaf, a 4-ml widemouth vial (14 mm OD, 45 mm high), closed with a size 15 rubber serum stopper, sleeve type, may be used. The $^{14}CO_2$ is generated inside this bottle with the leaf in place. After 1 minute the stopper and leaf are removed from the vial in a dry box, which is exhausted to a fume hood through a NaOH trap to remove the small amount of $^{14}CO_2$ remaining.

Exposures of algae and isolated chloroplasts. A small (about 10 ml), round-bottom glass flask with a tightly fitting serum cap may be used. Since the green material has a high coefficient for light absorption, a thin layer of biologic material on the bottom of the flask is desirable. This is achieved by gently swirling (by hand or mechanical shaker) 0.5 ml to 1.0 ml of the liquid suspension of cells or chloroplasts, which distributes the material in a thin layer on the bottom of the flask (8). Illumination may be provided from the bottom, preferably through a water bath with a transparent bottom to control temperature in the flasks.

With larger amounts of algae suspension, a flat-sided, disk-shaped vessel with stopcock-equipped inlets and outlet at top and bottom is used. The inside thickness of the vessel should be no more than 5 mm. Radioactive $H^{14}CO_3^-$ solution may be added through the top stopcock and samples taken periodically by opening first the top stopcock, and then briefly, the bottom stopcock. The vessel is held in a vertical position and illuminated from the sides, usually with incandescent lamps shining through infrared absorbing filters immersed in baths of cooling water. Much more elaborate systems for conducting kinetic experiments with algae exposed to radioactive tracers have been described (9). Such systems have provision for recirculating the mixtures of $^{14}CO_2$ in air through the algae and gas-monitoring instruments in a closed system, using a gas pump. Solenoid-operated valves permit rapid taking of samples of uniform size.

Chromatographic equipment. The simplest and cheapest equipment for two-dimensional paper chromatography consists of a widemouthed glass jar, such as that used in home fruit canning, equipped with a screw-on lid. Ascending chromatograms can be run in such jars by placing solvent in the bottom to a depth of about 2 cm and by setting a paper cylinder in the solvent, capping the jar, and allowing the solvent to travel up the paper by capillarity. To prepare the cylinder, the mixture to be analyzed is first dried on the 20 cm × 20 cm paper about 3 cm in from one corner, and the paper is rolled into a cylinder and the edges stapled together. After the first solvent reaches the top of the paper, the paper is dried, unstapled, rolled in the other direction, restapled, and placed in a second solvent for the second dimension. Unfortunately, this method does not give reproducibly good resolution of compounds compared to the resolution obtainable with descending chromatography and somewhat larger papers.

For small two-dimensional paper chromatograms (23 cm × 23 cm), a bench-top chromatography jar unit, about 30 cm × 30 cm square and 60 cm high, is available for about $125. This unit is supplied with a glass lid, adjustable stainless steel rack, and four solvent trough assemblies, each consisting of one glass solvent trough, one trough holder, two glass antisiphon rods, and one glass anchor rod. This assembly is satisfactory, provided the experiments can be run in a room with no more than 3 C variation in temperature. For rooms with larger temperature variation, more expensive insulated assemblies are required.

For large two-dimensional paper chromatography (which gives the best results and is used in research), Formica-lined cabinets with inside dimensions of about 40 cm × 65 cm × 60 cm may be purchased. Equipped with solvent assemblies, these boxes cost about $500. They may be easily modified for drying chromatographic

papers in situ after development. Two 10-cm round holes are made in the cover on the centerline of the long direction, with the edges of the holes about 6 cm apart. Each hole is equipped with a 10-cm air pipe that is flush with the inside of the lid and projects 5 cm on the outside. On the bottom of the lid, a 4 cm × 4 cm board, 30 cm long, is attached between the holes and crosses the short direction of the lid. To this board is attached a thin plate (30 cm × 30 cm) of stainless steel or other resistant material.

When chromatograms are being developed, both pipes are closed with large plugs. When the chromatograms are to be dried, the plugs are removed, and a flexible vent hose is attached to one of the pipes and led to an exhaust system. If the suction by the exhaust system is not sufficient, a closed air blower is placed in the exhaust line to move air from the box chromatographic cabinet to the exhaust system. Air enters the cabinet through the other pipe, is distributed through the cabinet by means of the plate attached to the bottom of the lid, and leaves by the exhaust system, drying the paper chromatograms in place. In this way, people are protected from excessive inhalation of the fumes of the volatile chromatographic solvents, and the papers do not have to be transferred while wet, thus minimizing the possibility of dropping and tearing.

Experimental procedures

Either of the experimental procedures for exposing plants to $^{14}CO_2$ may be combined with either of the chromatographic procedures.

Photosynthesis with leaves. Place a few crystals (about 10 mg) of citric acid in the bottom of a 4-ml widemouth vial. Cover the crystals with a thin layer (about 3 mm) of glass wool. Cut off a leaf from the alfalfa branch; weigh to 1 mg, and place the leaf in the vial, standing on its stem on the glass wool. Stopper the vial with the serum cap, and place in a "dry" box.

Carry out the following operations in a dry box vented to a fume hood through Ascarite (NaOH on asbestos). In this way, when the leaf is removed from the vial, the residual $^{14}CO_2$ will be trapped in the Ascarite and will not escape to the atmosphere.*

Insert a 2.5 inch, No. 20 hypodermic needle attached to a 0.1-ml graduated microsyringe into the stock solution of $NaH^{14}CO_3$ and draw 20 μl of the solution up into the microsyringe. Then suck in a few μl of air and carefully lay aside the syringe and needle on a piece of tissue in a tilted position *(needle point up)* so that the solution will not be spilled.

Hold the vial containing the leaf by the stopper and illuminate from the side with an incandescent lamp for 1 minute; remove from the light temporarily and insert the hypodermic needle through the serum cap until the tip touches the crystals of citric acid at the bottom of the vial. Be very careful during the insertion of the needle to not allow the solution in the syringe to flow out through the needle prematurely. *Practice this technique several times without radioactive solution.* When forcing the needle through the serum cap, take care not to push on the plunger of the syringe.

When the tip of the needle is in place, hold the vial in the light and inject the bicarbonate solution. Withdraw the needle and place it in a beaker containing dilute HCl. Continue the exposure of the leaf for 1 minute. (The time can be varied from one

* Federal and state regulations now place very low limits (0.1 microcurie per cu m of air) on the amount of $^{14}CO_2$ that can be legally released to the atmosphere.